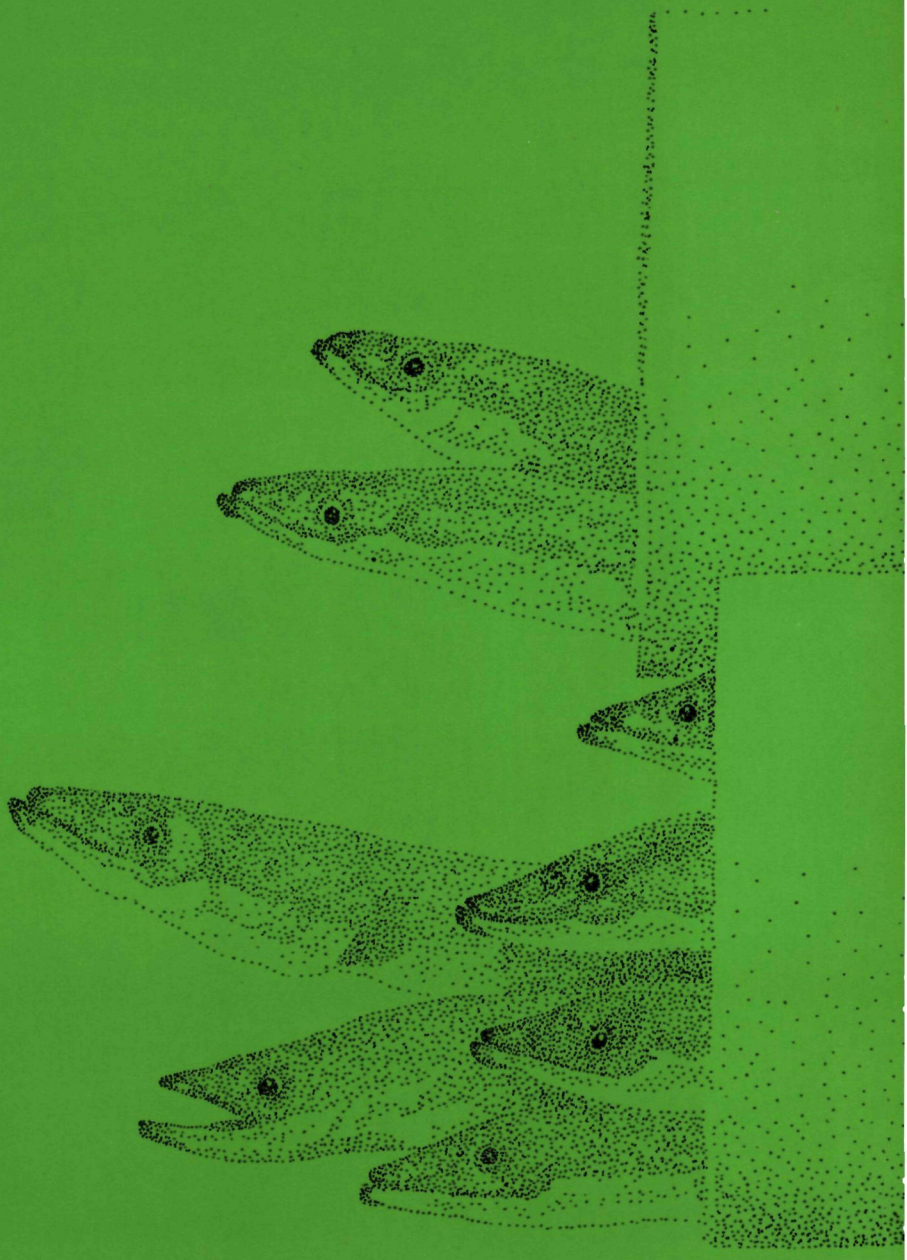
SYNTHESIS AND SECRETION OF THE CALCIUM REGULATING HORMONE STANNIOCALCIN IN TELEOST FISH



R.G.J.M. HANSSEN

SYNTHESIS AND SECRETION OF THE CALCIUM REGULATING HORMONE STANNIOCALCIN IN TELEOST FISH

een wetenschappelijke proeve op
het gebied van de natuurwetenschappen,
in het bijzonder de biologie

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CONTENTS

CHAPTER 1

General introduction

Hormonal regulation of calcium balance in vertebrates

Calcium is an essential element for the physiology of vertebrates. It is involved in major physiological processes such as muscle contraction, bone formation, blood coagulation, nervous impulse transduction, reproduction, and numerous enzymatic and membrane transport mechanisms. An appropriate regulation of calcium homeostasis therefore, is crucial for vertebrate life. Although this is equally true for both terrestrial and aquatic vertebrates, there are essential differences between these animal groups with respect to calcium physiology and its endocrine control.

Terrestrial vertebrates depend on calcium uptake via their food and thus calcium uptake is an episodic event. For maintenance of the calcium homeostasis of the extracellular fluid, bone is essential as an internal store for the deposition and remobilization of calcium. Other major organs playing a role in calcium regulation are the gut, involved in the uptake of calcium, and the kidney, which is important for calcium excretion and resorption.

Parathyroid hormone (PTH) and calcitonin are the main calcium-regulating hormones in terrestrial vertebrates. PTH keeps the calcium level of the extracellular fluid within narrow limits, directly by stimulation of calcium mobilization from the skeleton, and indirectly by controlling the synthesis of vitamin-D_3-metabolites that stimulate intestinal calcium uptake (Fraser and Kodicek, 1973; Bar and Hurwitz, 1980). Through these actions PTH prevents hypocalcemia. Calcitonin prevents hypercalcemia, in particular during episodes of high dietary calcium uptake, by stimulating calcium deposition in the skeleton (Talmage *et al*, 1981).

In fish, the skeleton does not act as a calcium store. This difference with terrestrial vertebrates is probably connected with the fact that fish are not, or only partially, dependent on the food for their calcium supply. In fish, the ambient water is the main source of calcium. Calcium uptake predominantly takes place via the gills and only to a small extent via the gut (Flik *et al*, 1985). The major actions of calcium-regulating hormones in fish are directed on the gills, although bone, gut and kidney are considered as additional targets (Pang and Pang, 1986).

Fish lack parathyroid glands and the pituitary gland is generally considered as the main source of hypercalcemic hormonal activity (Pang *et al*, 1973a). In many fish species the most probable hypercalcemic hormone is prolactin, which has hypercalcemic properties (Pang *et al*, 1973b; Wendelaar Bonga *et al*, 1984). The

calcium-regulating role of vitamin-D-metabolites has not been clearly defined but there is no doubt that these factors are involved in calcium metabolism (Fenwick *et al*, 1984). Another hormone with hypercalcemic properties is cortisol which induces hypercalcemia in salmonids (Björnsson *et al*, 1987; Flik *et al*, 1987). Despite its therapeutic use in human medicin (Austin and Heath, 1981), calcitonin has no clear anti-hypercalcemic potency in fish. Its role may be limited to stimulation of bone formation (Wendelaar Bonga and Lammers, 1982). Some authors have suggested that calcitonin has a hypocalcemic function but this is highly disputable. The corpuscles of Stannius are considered to be the source of the major hypocalcemic hormone in fish, stanniocalcin.

Isolation and characterization of stanniocalcin

The corpuscles of Stannius (CS), first described by Stannius (Stannius, 1839), are endocrine glands that exclusively occur in association with the mesonephric kidney of holostean and teleostean fish (Krishnamurthy, 1976; Wendelaar Bonga and Pang, 1986). The first indication for a possible hypocalcemic role of the CS in calcium regulation was found by Fontaine (Fontaine, 1964) who showed that operational removal of the CS (stanniectomy: STX) causes a hypercalcemia that can be alleviated by injection of a CS extract. On the basis of similar observations Pang *et al* (1974) proposed the name hypocalcin for the hypocalcemic CS principle. Hypocalcin was isolated and purified by Lafeber and colleagues who found that the major calcium regulating secretory product of the CS from rainbow trout was a 54 kDa glycoprotein, that proved to be dimeric in nature (Lafeber *et al*, 1988a).

Ten years earlier, Ma and Copp (1978) had isolated a 3 kDa peptide from chum salmon CS, which they called teleocalcin. Wagner *et al* (1986) gave the same name to a 39 kDa disulfide-linked oligomer that was isolated from the CS of sockeye salmon. Butkus *et al* (1987) reported for the australian eel the complete amino-acid sequence from the major CS glycoprotein with a calculated molecular mass of 24.632 Da.
The N-terminal amino-acid sequence of salmon, trout and eel CS products showed marked structural and functional homology (Butkus *et al*, 1987; Wagner *et al*, 1988; Lafeber *et al*, 1988a). To avoid further confusion about the name of the principal hypocalcemic product of the CS, several investigators decided to call it stanniocalcin.

Consensus about this new name was reached at the XIth International Symposium on Comparative Endocrinology in Malaga, Spain (1989).

Whereas parathyroid glands lack in fish it has been suggested that the CS might be their homologues. PTH-like effects of CS-extracts and purified trout stanniocalcin on bone resorption were demonstrated in a mammalian bioassay (Milet *et al*, 1979b; Lafeber *et al*, 1986; 1989). Moreover, bovine PTH was shown to induce hypocalcemia in fish bioassays (Wendelaar Bonga *et al*, 1986; Lafeber *et al*, 1988b). Milet *et al* (1980) reported the synthesis and secretion of a product by eel CS that was immunologically related to mammalian PTH. Further analysis of this CS product, called parathyrin (PCS), revealed a 32- to 34- kDa protein of which unfortunately no aminoacid sequence has been reported (Milet *et al*, 1989). Therefore, it remains unclear whether stanniocalcin and PCS represent the same hypocalcemic hormone. The reported similarity of the effects of PTH and stanniocalcin in some bioassays suggested homology between the structure of the hormones. However, no homology in primary structure could be demonstrated (Butkus *et al*, 1987). Similarities in the tertiary structure might explain the observed similar actions of both hormones (Lafeber *et al*, 1988b).

Aim of this study

The RIA- and ELISA-techniques necessary for stanniocalcin quantification have been developed only recently (Kaneko *et al*, 1988; Gellersen *et al*, 1988; Mayer-Gostan *et al*, 1991) and therefore little is known about the regulation of plasma stanniocalcin levels. Although plasma stanniocalcin levels may be altered by influencing stanniocalcin synthesis and metabolism, the regulation of stanniocalcin secretion will probably provide the most rapid control of plasma stanniocalcin levels.

In this study we investigated the synthesis of stanniocalcin (chapter 3) in relation to its anti-hypercalcemic role. Plasma calcium seems to be a dominant factor in the regulation of stanniocalcin secretion and, therefore, the chapters 2, 4, and 5 deal with the mutual relationship between plasma calcium and stanniocalcin. Chapter 6 deals with the kinetics of stanniocalcin in relation to its role in the control of stanniocalcin secretion. A possible neural cholinergic modulation of stanniocalcin secretion is described in chapter 7. In all chapters, the relevance of stanniocalcin regulation for its functional aspects in anti-hypercalcemic calcium regulation is a central point of discussion.

CHAPTER 2

Ionic and total calcium levels in the blood of the european eel (*Anguilla anguilla*): effects of stanniectomy and stanniocalcin replacement therapy

SUMMARY

Removal of the corpuscles of Stannius (STX) in the freshwater European eel causes a marked increase of blood ionic calcium as well as protein-bound calcium. The hypercalcemia peaks 20 days after STX and lasts at least another 20 days. In stanniectomized eels eel stanniocalcin decreased both blood ionic and total calcium concentrations. The reduction of plasma total calcium by stanniocalcin is attributed to a reduction in blood ionic calcium. We conclude that stanniocalcin regulates blood ionic calcium levels in fish.

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INTRODUCTION

The corpuscles of Stannius (CS) of holostean and teleostean fish produce an important hypocalcemic hormone. For a variety of teleost species it has been shown that removal of the CS (stanniectomy, STX), results in hypercalcemia (Fontaine, 1964; Butler, 1969; Chan, 1970; Pang, 1971a; Fenwick, 1974; Wendelaar Bonga and Greven, 1978; Urasa and Wendelaar Bonga, 1987). This hypercalcemia was found to be transitory (Fontaine, 1967; Chan, 1970; Fenwick, 1978). Injection of CS extracts (Fontaine, 1964; Pang, 1971a; Kenyon *et al*, 1980) or homotransplantation of the corpuscles (Fenwick and Forster, 1972; Pang, 1973) restored normocalcemia. Contradictory results have been reported with respect to the regulation by the CS of other plasma electrolytes such as Na^+, K^+, Mg^{2+}, PO_4^{2-}, and Cl^-. Presumably, the CS are not directly involved in the regulation of these ions (Chan, 1972; Pang *et al*, 1975; Fenwick, 1985).

Although a negative feedback between CS and plasma ionic calcium has been suggested (Bailey and Fenwick, 1975; Aida *et al*, 1980; Urasa and Wendelaar Bonga, 1987) evidence has been circumstantial. In their experiments with frog hearts McLean and Hastings (1935) provided evidence that ionic calcium is the major physiologically active form of calcium in the body. This is now generally accepted (Heining *et al*, 1983). Calcium in blood or plasma can be subdivided in a protein-bound and an ultrafiltrable (or dialytic) fraction, which consists of calcium complexed to anions and of ionic calcium. For the freshwater European eel it was reported that about 70% of the plasma calcium is ultrafiltrable, 54% being ionized (Chan and Chester Jones, 1968). The plasma ionic calcium concentration in the freshwater eel appears rather constant (concentrations reported range from 1.3 to 1.6 mM; Chan and Chester Jones, 1968; Chan, 1972; Fenwick, 1974) and comparable with values found in mammals.

In this report we re-evaluated calcium levels in blood of the European eel, using a Radiometer ionized calcium analyzer. Eels were cannulated to allow serial stress-free sampling. Blood ionic and total calcium levels were manipulated by stanniectomizing eels or by stanniocalcin replacement.

MATERIAL AND METHODS

Animals

Sexually immature freshwater eels (*Anguilla anguilla*), weighing about 300 g, were purchased from a commercial fish dealer in The Netherlands. Upon arrival in the laboratory the fish were kept unfed in 300 l well-aerated tanks supplied with running tapwater (total calcium 0.7 $\pm$ 0.1 mM). The temperature of the water was approximately 12 °C. Before surgery the eels were acclimated to these conditions for at least 14 but no longer than 42 days.

Preparation of eel CS extract and eel hypocalcin

Freshly dissected eel CS were homogenized in ice cold saline (0.6% sodium chloride) and the homogenate was centrifuged (5 min. at 9000 x g). The supernatant (CS extract) was immediately injected as described below. Eel stanniocalcin was isolated using concanavalin-A affinity chromatography (Lafeber *et al*, 1988a). The CS material without affinity to concanavalin-A is referred to as residue and was devoid of stanniocalcin as judged by sodiumdodecylsulphate polyacrylamide gelelectrophoresis (Laemmli, 1970). Doses injected per 100 g fish were the extract of 6 mg fresh eel CS (6 fish equivalents), 50 μg eel stanniocalcin (8 fish equivalents), or 80 μg residue protein (4 fish equivalents); the carrier volume was 200 μl saline/100 g fish. Saline injections served as controls.

Surgery

Eels were anaesthetized in ethylaminobenzoate (MS222, 2.5 g/l, pH 7.8) and stanniectomized or sham-operated as described by Leloup-Hatey (1964). Muscle and skin were sutured carefully and the fish were allowed to recover in 100 l tanks supplied with aerated running tapwater. The CS were used for replacement studies.
The pneumogastric artery was cannulated as described by Chester Jones *et al*, (1966). Cannulated eels were housed individually in opaque cylindrical containers supplied

with running tapwater. Between sampling procedures the cannula was filled with a saline (0.6%, pH 7.8) containing polyvinyl pyrrolidon (0.12 g/ml) and sodium heparin (500 U/ml), and closed with a metal pin. The eels were allowed 2 days of recovery from surgery. The set-up allowed free movement of the eels, stress-free blood sampling, and intra-arterial injection.

Experimental protocols

A period of 17 days maximally was found appropriate for serial blood sampling. Therefore, to cover a period of 40 days, three groups of 16 eels were used to study the long term effects of stanniectomy. In each group 8 eels were stanniectomized and 8 sham-operated. The first group was cannulated two days before, the second and third group 11 and 26 days after surgery, respectively. Daily, a 200 μl blood sample was taken. To assess any interference of the cannulation and blood sampling procedures with blood calcium levels, in one experiment blood was sampled by cardiac puncture from two separate groups of eels 40 days after STX or sham-operation, respectively. Blood calcium values in STX or sham-operated fish were not effected by these procedures. In STX fish values were elevated and comparable with values in cannulated STX fish.

CS extract and stanniocalcin was injected in STX eels, as a more pronounced blood calcium lowering effect may be anticipated in STX rather than intact eels (Kenyon *et al*, 1980). Eel CS extract or eel stanniocalcin were tested in cannulated eels. On day 15 after STX, between 8-10 a.m., a single hormone injection was given. Before injection a 500 μl blood sample was taken, 200 μl used for analyses, and the remaining 300 μl re-injected immediately following the hormone injection. This ensured that all hormone entered the blood stream. Blood samples were collected for 3 days.

Plasma analyses

Blood samples were collected in 1.5 ml eppendorf cups containing 5 μl calcium heparin (Radiometer). Blood ionic calcium and pH were determined with an ionic calcium analyzer (ICA-1, Radiometer). The remaining blood was separated in plasma and cells by centrifugation (1 min at 9000 x g). Plasma total calcium, magnesium, and protein were estimated with commercial kits (calcium reagent kit, Sigma; magnesium reagent kit, Sigma; protein reagent kit, with BSA as a reference, Biorad). Plasma osmolality was measured with a micro-osmometer (Roebling) using distilled water and a 300 mOsmol/kg solution (Sigma) as standards. Measurements of ionic calcium in whole blood reflect plasma values (Andreasen, 1985). Indeed we did not observe a significant difference between Ca^{2+} concentrations in blood or plasma.

Calculations and statistics

The data are presented as means $\pm$ S.E.M. The Mann-Whitney *U*-test (one-tailed) was used for statistical evaluation. Significance was accepted at $P < 0.05$. For the analysis of the effects of STX, values of STX-eels were compared with corresponding values of sham-operated eels. In replacement studies values of hormone injected eels were compared with corresponding values for saline injected eels.

Table 1. *values for haematocrit (Hct), blood pH, plasma osmolality, plasma protein and plasma magnesium concentration in 14-day stanniectomized and sham-operated eels. Values are presented as means $\pm$ S.E.M. Numbers in parentheses indicate N.*

	Hct (%)	Blood pH	Osmolality (mosmol kg^{-1})	[Protein] (mg ml^{-1})	[Mg] (mmol l^{-1})
STX	23 $\pm$ 2 (7)	7.81 $\pm$ 0.02 (40)	277 $\pm$ 8 (7)	39 $\pm$ 1 (17)	1.42 $\pm$ 0.12 (5)
Sham	22 $\pm$ 2 (5)	7.84 $\pm$ 0.01 (33)	288 $\pm$ 4 (6)	43 $\pm$ 2 (8)	1.41 $\pm$ 0.09 (7)

RESULTS

In 14 days stanniectomized eels haematocrit, blood pH, plasma osmolality, plasma magnesium concentration, and plasma protein concentration are unchanged (Table 1). STX causes an increase of ionic and total calcium (Figures 1A, B, respectively). The rise in total calcium surpasses the rise in ionic calcium. Hypercalcemia reaches its maximum around 20 days after STX. Total and ionic calcium in 40 days STX eels are still significantly elevated and are comparable to values in 20 days STX eels.

Figures 2A-D summarize replacement studies in 15 days STX eels. Pre-injection values are 3.03 ± 0.15 mM for blood ionic and 5.77 ± 0.20 mM for plasma total calcium ($n = 37$; Figure 1A, B). Injection of eel CS extract (Fig. 2A) and eel stanniocalcin (Fig. 2B) caused a decrease of blood ionic and plasma total calcium while injection of residue (Fig. 2C) or saline (Fig. 2D) had no significant effects. Blood total calcium in eels injected with CS extract had decreased significantly 2 hours after injection; plasma ionic calcium 3 hours after injection. Blood ionic and plasma total calcium in hypocalcin injected eels had decreased significantly 3 and 5 hours after injection, respectively. Maximum effects on both ionic and total calcium levels were found 24 and 48 hours after injection, respectively. Reductions in blood calcium levels lasted up to 72 hours after hormone injection.

DISCUSSION

Our experiments indicate that stanniocalcin controls blood Ca^{2+} levels. Also non-endocrine factors may effect calcium levels in blood, in particular CO_2, pH, and temperature. Storage (up to 2 hours) of our samples under aerobic conditions did, however, not effect blood pH and Ca^{2+} levels. We conclude therefore that the Ca^{2+} level in eel blood is not directly influenced by CO_2 levels, as it is in terrestrial vertebrates. This seems in line with the pH buffer mechanisms in fish blood, where pH is regulated mainly by plasma bicarbonate adjustment instead of variations in CO_2 ventilation (Hôbe *et al.* 1984). In human blood a change in pH of 0.1 unit corresponds to a change in ionic calcium of approximately 0.06 mM (Fogh-Andersen, 1981; Brauman *et al.* 1983). In trout, however, the pH effect on ionic calcium is 3-6 times weaker (Andreasen, 1985). We conclude that changes, if any, in blood pH in our procedures have gone undetected. Since our automated ionic calcium analyzer is

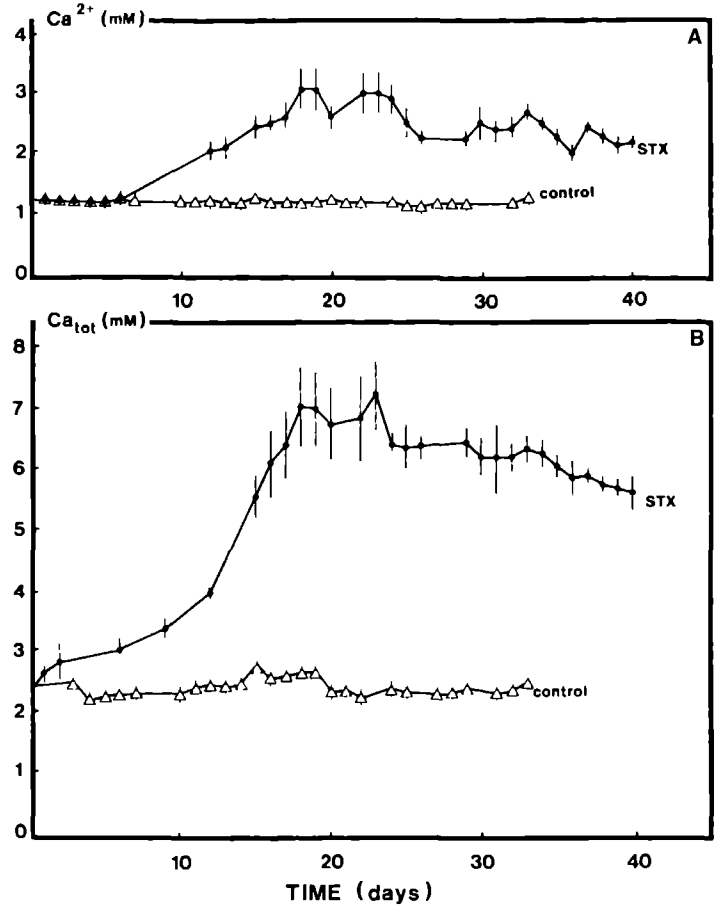

Fig. 1. *Effect of stanniectomy (STX) or sham-operation (control) on the blood ionic calcium concentration (A) and blood total calcium concentration (B) in cannulated eels. Values are presented as means ± S.E.M. (N = 8).*

operated at 37 °C, the ionic calcium level measured is not the actual *in vivo* ionic calcium concentration. In human blood, temperature is reported to effect directly the protein binding of ionic calcium or indirectly via a temperature induced change of pH

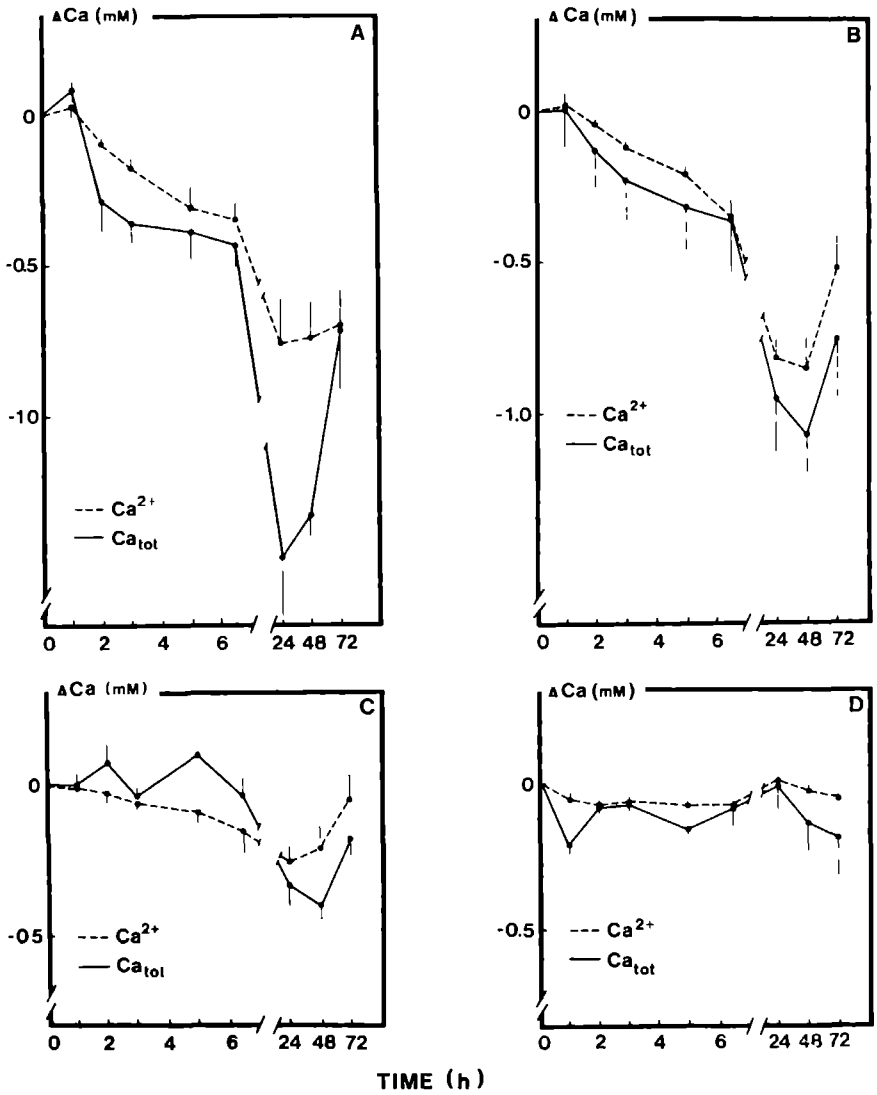

Fig. 2. *Effect of stanniocalcin replacement therapy on blood ionic (---) and total (—) calcium concentration in cannulated eels, 15 days after stanniectomy. Fish were injected with eel CS extract (A), eel stanniocalcin (B), eel residu (C) or saline (D). Values are presented as means ± S.E.M. (N = 11) and are shown as differences compared with preinjection levels.*

(Heining, 1983). A rise in temperature will cause more calcium to bind to protein and as a result ionic calcium levels will decrease; concomitantly a rise in temperature will evoke a pH drop which in turn causes a rise in Ca^{2+} levels. In human blood the overall temperature effect on Ca^{2+} levels is an increase of approximately 0.002 mM/°C (Heining, 1983). Assuming a similar relationship for eel blood - the relationship between blood pH and temperature of trout blood is similar to that of human blood (Perry *et al.* 1985) - a 25 °C increase in eel blood temperature would increase blood Ca^{2+} by 0.05 mM. This deviation is within the error of our measurements.

Our observation that STX increases total calcium in the plasma of eels is in agreement with reports in the literature on a variety of species (Fontaine, 1964; Butler, 1969; Pang, 1971a; Chan, 1972; Fenwick, 1974; Wendelaar Bonga and Greven, 1978; Urasa and Wendelaar Bonga, 1987). The hypercalcemia was not caused by an increased bone demineralization (Lopez, 1970) but resulted from an increased net branchial calcium uptake that was already obvious 7 days after STX (So and Fenwick, 1977; Milet *et al.* 1979a). We show here that blood ionic and plasma total calcium levels rise concomitantly after STX. Both blood ionic and plasma total calcium levels reach maximum values 20 days after STX and stay elevated at least another 20 days. This observation contrasts with reports by Fontaine (1967), Chan (1972), and Fenwick (1978), who found that plasma total calcium levels return to normal 56, 42, or 35 days after STX, respectively. The authors suggested that activation of the ultimobranchial bodies after STX may have resulted in the restoration of normocalcemia. Reports on the effect of calcitonin on plasma calcium levels in fish are contradictory. Hypocalcemic effects of mammalian or fish calcitonins in fish (Chan *et al.* 1968; Wendelaar Bonga, 1981) are at all doses tested minor and such effects have not always been confirmed by others (Pang, 1971b). The present results indicate that the ultimobranchial bodies are unable to restore normocalcemia after STX under our experimental conditions, and confirm that the hypocalcemic potency of these glands is low when compared to that of the CS.

The increase in blood ionic calcium after STX does not completely account for the increase in plasma total calcium, which implies that the protein-bound calcium level also rises after STX. This observation is consistent with earlier observations by Pang (1971a) and Chan (1972) who found that both dialytic and non-dialytic calcium contribute to the increase of total plasma calcium in stanniectomized killifish and eel, respectively. We did not measure a rise of blood ionic calcium in the first 6 days after STX as reported by Chan (1972). Plasma total calcium however did increase during

the first 6 days after STX. This observation is in good agreement with observations by Fenwick (1974) that plasma dialytic calcium in the American eel is unchanged on the fifth day after STX. A significant increase in blood ionic calcium was observed 12 days after STX and on. Fenwick (1974) reported that the dialytic calcium fraction was elevated 10 days after STX. Since the hypercalcemia following STX is caused by an increased net branchial uptake of Ca^{2+} (So and Fenwick, 1977; Milet *et al.* 1979a), one would expect a rise of plasma ionic calcium. Since only protein-bound calcium increases in the first days after STX, we conclude that the increased Ca^{2+} influx in the blood is buffered by binding to plasma proteins. Increased binding of ionic calcium to plasma proteins may result from an increase in plasma protein concentration or from a change in the Ca^{2+} binding capacity of plasma proteins. Chan (1972) speculated that the appearance of a calcium binding protein could explain the rise in non-dialytic calcium in 14 days STX Japanese eels. However, we did not find that the rise of the blood calcium concentration after STX was accompanied by a rise of the plasma protein concentration. The maximum binding capacity for blood ionic calcium in human blood is approximately 0.12 mmol Ca^{2+}/g protein, 90 % being bound to albumin (Pedersen, 1972). If we assume a similar maximum binding capacity for eel blood, and take into account a protein concentration in eel plasma of 40 mg/ml, it follows that in untreated and sham-operated eels ($[Ca^{2+}]$=1.25 mM, $[Ca_{tot}]$=2.50 mM) the binding capacity amounts to 0.03 mmol Ca^{2+}/g protein. During STX the binding capacity increases until a maximum value of 0.11 mmol Ca^{2+}/g protein is reached 21 days after STX ($[Ca^{2+}]$=2.50 mM, $[Ca_{tot}]$=7.00 mM). This is further reflected by the decrease in the Ca^{2+}/Ca_{tot} ratio in blood which is around 0.50 in untreated and sham-operated eels and which decreases to 0.40 in STX eels. We conclude that the increase of Ca^{2+} taken up during the first days of STX is buffered by increased binding to plasma calcium binding proteins. This might be due to a qualitative change of plasma proteins.

Injections with homogenates of CS obtained from different teleost species have been reported to decrease total plasma calcium levels in STX fish (Fontaine, 1964; Kenyon *et al.* 1980), as well as in intact fish adapted to low calcium water (Pang *et al.* 1981; Wendelaar Bonga *et al.* 1986). So and Fenwick (1979) showed that CS extract injected in STX eels reduced the branchial calcium uptake. Recently Lafeber *et al.* (1988c) concluded that in rainbow trout the reduction of plasma calcium, caused by injection of CS extract, resulted from a stanniocalcin induced reduction of whole body Ca^{2+} uptake. The experiments presented in this paper show a decrease of blood ionic as

well as plasma total calcium after injection of eel CS extract or eel stanniocalcin in stanniectomized eels. The hypocalcemic effect can be observed for more than 48 hours. To our knowledge there are no other peptide hormones that can exert their function for such a long period. We observed a decrease of blood ionic calcium that almost fully accounts for the decrease of blood total calcium. Bailey and Fenwick (1975) observed a decrease in ionic calcium and not in total calcium after injection of eel CS homogenates in intact eels. They suggested an influence of the CS extract on calcium binding to plasma proteins. Our experiments do not support their suggestion since no increase of the calcium binding capacity of plasma proteins was found. At the time of injection, 15 days after STX, the binding capacity amounts to 0.06 mmol Ca^{2+}/g protein ($[Ca^{2+}]$=3.03 mM, $[Ca_{tot}]$=5.77 mM, [protein]=40 mg/ml). Since the reduction of ionic calcium equals the reduction of total calcium after stanniocalcin replacement therapy, no change in the binding capacity will occur. The rather instantaneous effect of stanniocalcin on the elevated blood ionic calcium level in STX eels indicates that stanniocalcin exerts its hypocalcemic action mainly by inhibition of the Ca^{2+} uptake. This inhibition results in a net calcium efflux as has been shown by Lafeber *et al.* (1988c).

CHAPTER 3

Rainbow trout corpuscles of Stannius: stanniocalcin synthesis *in vitro*

SUMMARY

In freshwater rainbow trout, *Oncorhynchus mykiss* (formerly called *Salmo gairdneri*), experimentally induced hypercalcemia results in release of immunoreactive stanniocalcin from the corpuscles of Stannius (CS) and stimulates synthetic and releasing activities of the glands as measured *in vitro*. 'Pulse-chase' experiments showed that stanniocalcin (STC) is a 56 kDa glycoprotein, processed from a 64 kDa precursor, prostanniocalcin (PSTC). PSTC and STC are homodimeric molecules that are readily split into monomers in the presence of reducing agents such as 2-mercaptoethanol. The monomeric form of PSTC and STC contains an approximately 5-6 kDa glycomoiety. Neither this sugar residue nor the N-terminal amino acid sequences of PSTC or STC proved to contain antigenic sites for the antiserum used in this study. Two-dimensional gel electrophoresis indicated the presence of several isoforms of PSTC and STC molecules, that may reflect different stages of maturation of the (pro)hormone.

G. Flik, T. Labedz, J.A.M. Neelissen, R.G.J.M. Hanssen, S.E. Wendelaar Bonga and P.K.T. Pang
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INTRODUCTION

Corpuscles of Stannius (CS) are endocrine glands unique for holostean and teleostean fish; they produce the predominant hypocalcemic hormone in fish (Fontaine, 1964), stanniocalcin (STC). Calcitonin, although first purified from fish ultimobranchial glands (Copp *et al.* 1967), plays an enigmatic and probably insignificant role in fish plasma calcium homeostasis. Upon ultrastructural examination the STC producing cells of the CS of the euryhaline tilapia, *Oreochromis mossambicus*, kept in fresh water (0.8 mM Ca^{2+}) differ from those of tilapia kept in calcium-enriched fresh water (5 mM Ca^{2+}) in that they show a lower degree of granulation and more extended Golgi areas and granular endoplasmic reticulum (Urasa and Wendelaar Bonga, 1987). Such observations are in line with the assumption of an increased need for STC when the fish is exposed to high-calcium media. because tilapia, *Oreochromis mossambicus*, in high-calcium medium had slightly elevated plasma calcium levels, it is the calcemia which is thought to control the activity of the CS. Indeed, this notion has successfully been used to experimentally degranulate the CS *in vivo* by calcium injection (Lopez *et al.* 1984; Lafeber *et al.* 1988a).

Only recently reports have been published on the isolation of STC. Although consensus exists on the glycoproteinaceous nature of the hormone (concanavalin-A affinity chromatography forms the basis for its isolation), molecular radii reported for STC's of several species vary from 39 kDa in salmon (Wagner *et al.* 1986) to 54 kDa in rainbow trout (Lafeber *et al.* 1988a). A dimeric nature of the STC molecule has been postulated, as the use of reducing agents yields a molecule approximately half the size of native STC (Lafeber *et al.* 1988a). Amino acid sequence analysis of STC preparations (which are carried out under reducing conditions) consistently yield single N-terminals (Wagner *et al.* 1986; Lafeber *et al.* 1988a), although for rainbow trout STC a microheterogeneity at position 7 (Ser/Glu) was reported (Lafeber *et al.* 1988a). Butkus and coworkers (1987) purified a 32 kDa protein (in its reduced form) from CS of the Australian eel and cloned its cDNA. The N-terminal amino acid sequence of this protein shows a high degree of homology with that of salmon and trout STC. Interestingly, the reduced (and therefore supposedly monomeric) Australian eel STC molecule contains 15 half-cystines, which is a high incidence of this amino acid (total sequence 231 amino acids) and allows for disulfide-bridged subunits in the native hormone.

This report deals with studies on STC synthesis by trout CS in vitro. 'Pulse' and 'pulse-chase' experiments, concanavalin-A adsorption, immunoprecipitation-, immunoblot- and ELISA-techniques, and one- and two-dimensional gel electrophoresis were performed to elucidate the nature of newly-synthesized STC. The effect of experimental hypercalcemia on CS synthetic activity *in vitro* was included in these investigations.

MATERIAL AND METHODS

Animals

Rainbow trout, *Oncorhynchus mykiss*, of both sexes ranging in body weight from 200 to 700 g, were kept indoors under a photoperiod of 16 h light alternating with 8 h darkness in 1000 l tanks supplied with well-aerated, dechlorinated and filtered City of Edmonton tapwater. The water temperature was held at 10 ± 1 °C, the water pH was 7.4 and the Ca content 0.85 ± 0.12 mM. The fish were fed daily with Purina trout pellets. Trishydroxymethyl aminomethane (Tris)-buffered (pH 7.4) 3-aminobenzoic acid ethyl ester (MS 222, Sigma; 0.5 $g.l^{-1}$) was used as an anesthetic; the fish were killed by transection of the spinal cord.

Analytical procedures

Plasma total calcium was determined with a commercial calcium kit (Sigma); combined calcium/phosphate standards were used as a reference. Protein was estimated with a commercial reagent kit (Biorad) using bovine serum albumin (BSA, Biorad) as reference.

Effects of calcium on CS synthetic activity

To provoke an experimental hypercalcemia, fish were injected intraperitoneally with $CaCl_2$ (0.34 mmol.kg^{-1} per day, injection volume 1 ml.kg^{-1}) for three consecutive days. Injections of NaCl solution of identical molarity served as control. Four hours after the last injection, a blood sample was taken by puncture of the vessels of the caudal peduncle, using a heparinized syringe with a 23-gauge needle. Cells were separated from plasma by centrifugation (15 s, 9000 **g**) and the plasma stored at -20 °C until further analysis. The CS were removed and prepared for incubation (see below).

Pulse and pulse-chase incubations

Immediately after the fish had been killed, the CS were removed and collected in Hanks' balanced salt solution (HBSS; Sigma, H13387). Next, adhering renal tissue was carefully removed using a binocular microscope, and the connective tissue capsule of the CS incised. CS tissue was pre-incubated for 60 min in 500 μl HBSS at 22°C. Subsequently, the tissue was transferred to 100 μl HBSS containing 1.85 to 3.70 MBq of the radiolabeled amino acid of choice. The [^{35}S]-labeled amino acids used were (specific activity in GBq.mmol^{-1}): cystein (4.58) and methionine (11.66). For labeling of glyco-moieties of glycoproteins, D-[6-^{3}H]-glucosamine (0.42) was used. Labeled glucosamine was purchased from Amersham PLC., the labeled amino acids were from New England Nuclear.

In pulse-chase experiments, a 1 h pulse was followed by a chase period of increasing duration in Medium 199 based on HBSS (Sigma, M0393). At the end of pulse and chase incubation the tissue was carefully rinsed in Medium 199 (three times 500 μl). The tissue was homogenized in 1 ml 50 mM acetic acid (HAc) in a tight all-glass homogenizer; the homogenate was centrifuged in an Eppendorf minifuge at 9000 **g** for 10 min. An aliquot of the supernatant (further called "extract") was used to determine protein content and the remainder lyophilized and stored at -80°C until further analysis.

Immunoprecipitation and concanavalin-A absorption

Indirect immunoprecipitation of STC antigen was carried out strictly following the procedure described by Anderson and Blobel (1983), using a previously characterized antiserum for trout STC (RADH I; Kaneko *et al.* 1988); Wendelaar Bonga *et al.* 1989) and Protein A Sepharose CL-4B (Pharmacia) as a solid-phase immunoadsorbent. Lyophilized CS extract was reconstituted with distilled water; sodium dodecyl sulphate (SDS) was the detergent in the immunoprecipitation procedure.

Glycoprotein isolation was carried out using Concanavaline-A Sepharose (Pharmacia) as a solid-phase adsorbent. Reconstituted CS extract was mixed overnight with 10 volumes Con-A Sepharose in "Con-A buffer", which consisted of 15 mM Tris-HCl (pH 7.4) containing 1mM each $MnCl_2$, $MgCl_2$ and $CaCl_2$, and 1 M NaCl (Lafeber *et al.* 1988a). Products without affinity for Con-A were removed by 5 washes with Con-A buffer, precipitating the Con-A sepharose by centrifugation (15 s, 9000 **g**). The material bound to Con-A was dissociated from the lectin by Con-A buffer containing 0.3 M α-methyl-D-glucoside and precipitated with 10% trichloroacetic acid.

Removal of carbohydrate moieties

Extract of CS, that had been incubated for 4 h in [^{35}S]-cystein, was denatured in 20 μl 0.5% SDS and 0.1 M 2-mercapto-ethanol in water and divided in two 10 μl portions (each equivalent to 5 μg BSA). To each portion 10.8 μl 0.55 M sodium phosphate buffer (pH 8.1), 5 μl 7.5% Triton X-100, and 4.2 μl water (control) or glycopeptidase F solution (EC 3.2.2.18, PNGase F, Boehringer; 0.4 U) was added. After overnight incubation at 37°C, samples were brought to 48 μl with water and 2 μl 25% SDS was added; the samples were microwave-cooked (4 min) to stop enzymic activity and further processed for immunoprecipitation and separation.

Separation techniques

For the estimation of the relative molecular weight of the CS products, samples were separated by sodium-dodecyl sulphate polyacrylamide slab gel electrophoresis (SDS-PAGE) using a Biorad Protean II or Mini Protean II Slab Cell, following the protocol of Laemmli (1970); where indicated however, reducing agents were omitted from the sample buffer. As markers for molecular weight were used "low molecular weight standards" (Biorad, cat. no. 161-304) when gels were stained with Coomassie Blue. For fluorographs, prestained SDS-PAGE standards (Biorad, cat. no. 161-0305) were mixed with [^{14}C]-methylated protein markers (Amersham PLC., CFA.626).

Two-dimensional gel electrophoresis was essentially performed as originally described by O'Farrel (1975), using Biorad's Mini-PROTEAN II 2-D cell. The isoelectric focussing (IEF) gel was run between pH 5 and 8, and the second dimension was a 10% acrylamide gel. IEF was for 12 to 16 h at a constant 500 V. Upon completion of the IEF run, the gel-rod was applied directly to the second dimension without equilibration, and overlayed with 200 μl SDS-PAGE sample buffer. The pH gradient in the IEF gels was estimated using a "high pI calibration kit" (Pharmacia).

Western blots

One- or two-dimensional (SDS-PAGE) gels (1 mm thickness) were equilibrated for 15 min in 25 mM Tris/192 mM glycine (pH 8.3) containing 20% methanol. Electrophoretic transfer of products was considered complete after 1 h at 100 mA (no products detectable by silverstaining in blotted gel). The blots were immunostained as follows. After a 15 min preincubation of the blot in 3% chicken egg albumin (CEA) plus 1% normal goat serum (NGS) in phosphate buffered saline (PBS, 10 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl) at room temperature, the blots were incubated with RADH-I (dilution 1:5000) in PBS containing 1% CEA and 1% NGS for 15-17 h at 4°C. Next, the blot was rinsed 4 times 5 min in PBS containing 1% CEA at room temperature, followed by a 2 h incubation at room temperature with the second antibody/peroxidase complex (GARPO, dilution 1:2000; Nordic) in PBS containing 0.1% CEA and 1 % gelatin. Subsequently the blot was rinsed 2 times 5 min and 2 times 10 min in PBS containing 0.1% CEA. The peroxidase enzymic activity was visualized by adding 0.0005% 4-chloronaphtol plus 0.004% H_2O_2 and 17% methanol

in 50 mM Tris/HCl (pH 7.6). The reaction was stopped by transfer of the blot to plain water.

Staining and fluorography

Gels were fixed in 40% methanol:10% acetic acid (HAc) in water (1 h), 5% methanol:7% HAc in water (1h) and 10% glutaraldehyde (BDH) in water (30 min) and rinsed for at least two hours in flowing demineralized water. Staining was performed in the first fixative, containing 2 $g.l^{-1}$ Coomassie Blue R-250 (Biorad). The gels were destained in the same aqueous solution of methanol and HAc. For fluorography the fixed gels were impregnated with PPO/POPOP according to the method of Bonner and Laskey (1974). Before drying (Biorad slab dryer), the gels were dehydrated for 8 to 12 h in 50% methanol in water containing 3% glycerol. Preflashed Kodak XAR-5 X-ray film was used; exposure time was 2 to 72 h at -80 °C. In some cases gels were sliced (2 mm) and the radioactivity in the gel determined by liquid scintillation counting. To this end, 0.5 ml H_2O_2 was added to the gel slice, followed by incubation for 12 h at 60 °C. Next 4 ml Scintiverse (Fisher) was added and the radioactivity determined in a LKB Rackbeta LSC, equipped with a dpm program. Stained gels and fluorographs were scanned densitometrically with a LKB 2202 Ultroscan Laser Densitometer equipped with a LKB 2220 Recording Integrator.

ELISA

To determine total STC antigenic activity in CS extracts, an ELISA was used that has been described in detail (Kaneko *et al.* 1988). Microtitration plates were coated with 200 μl of serial dilutions of purified trout STC (Lafeber *et al.* 1988a) or tissue extract. The RADH-I antiserum dilution was 1:5000. Alkaline phosphatase conjugated IgG (1:2000, Miles Scientific) was the second antibody. Alkaline phosphatase activity was determined on the basis of para-nitrophenyl phosphate hydrolysis, determined spectrophotometrically as the change in A_{405}. All samples were assayed in triplicate.

Calculations and statistics

Values are expressed as means ± standard deviation, unless otherwise indicated. Linear regression analysis was based on the least squares method. Differences between means were statistically evaluated with the Mann-Whitney U-test. The fiducial limit was $P < 0.025$.

RESULTS

As shown in Figs. 1 and 2 by Western blot analyses, the antiserum used in our studies recognizes a 32 (prostanniocalcin, PSTC) and a 28 (STC) kDa M_r species, both with pI-values between 5.6 and 7.2. The purified STC preparation consists for the major part of a 28 kDa product and is slightly contaminated with a 32 kDa product. The pI-values of the products in the purified hormone preparation coincide with those recognized by the RADH I antiserum in the CS homogenate.

During a 1 h pulse incubation in HBSS supplied with [^{35}S]-cystein a 32 kDa M_r species is formed as the major product. This radioactive product comigrates with the minor 32 kDa peak revealed by CBB staining of the gel (Fig. 3 left panel). In the absence of reducing agents that are normally applied in the SDS-PAGE procedure (Kaneko *et al.*, 1988), the [^{35}S]-cystein labeled product appears as a 64 kDa M_r species; the major CBB-stained product is a 56 kDa M_r species (Fig. 3 right panel).

As shown in Fig. 4, the predominant 32 kDa M_r species synthesized by the CS during a 1 h pulse incubation in [^{35}S]-methionine supplied HBSS has almost completely converted into a 28 kDa M_r species over a 6 h chase period in medium M199. We will further refer to these M_r species as PSTC and STC, respectively (see DISCUSSION).

It has been published before that the amount of CS tissue in adult freshwater rainbow trout is linearly related to body weight and comes to approximately 10 mg wet weight per kg fish (Lafeber *et al.* 1988a). The amount of protein extracted (P_{ext}) from trout CS is linearly related to the wet weight of the tissue (W_{cs}, in mg) according to: $P_{ext} = 36.14 (\pm 1.15) W_{cs} - 1.94 (\pm 3.71)$ μg BSA equivalents ($r_o = 0.995, n = 42, P < 0.001; 8$). As determined by ELISA, the extracted protein consisted of $28.3 \pm 2.6\%$ ($n=9$) of immunoreactive STC (irSTC), a value close to the 20% estimated on the basis of densitometric analysis of silverstained SDS-gel electrophoretographs of the same tissue (Lafeber *et al.* 1988 a). These data taken together indicate that the amount

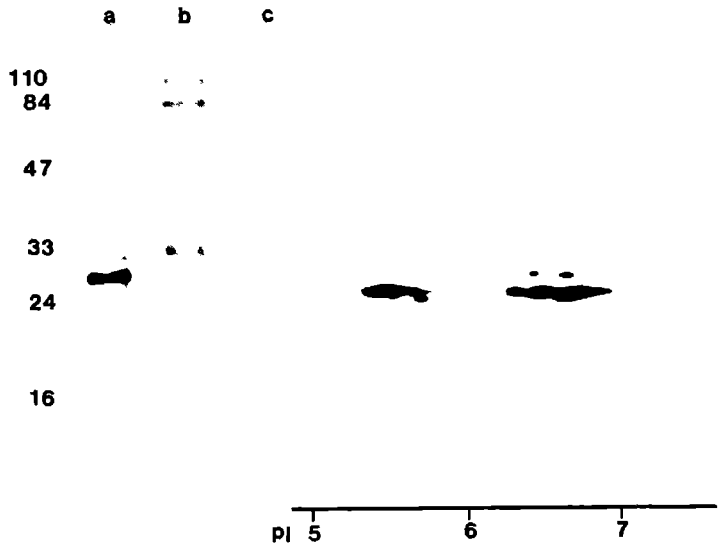

Fig. 1. *Western-blot analysis of corpuscles of Stannius (CS) extract (2 μg protein). Samples were separated by 1- and 2-dimensional gel electrophoresis under reducing conditions. Transfer to nitrocellulose was complete after 1 h at 100 V. Nitrocellulose was probed with a 1:5000 diluted rabbit anti-Dutch hypocalcin hormone, first bleeding (RADH I) antiserum, followed by peroxidase-conjugated goat anti-rabbit globulin. a: One-dimensional SDS-PAGE (polyacrylamide gel electrophoresis); b: markers; c: two-dimensional SDS-PAGE. pI values of first dimension are indicated. Major products are observed in 32- and 28-kDa region. Apparent molecular mass of marker proteins are indicated in kDa on left.*

of irSTC stored in the CS of trout increases with increasing body weight and that the STC content of the CS may be predicted by linear extrapolation on the basis of fish weight or CS extractable protein. The CS irSTC content was negatively correlated (r_o = -0.874, n = 8, P < 0.01) with the plasma total calcium concentration (Fig. 5), confirming preliminary data published recently (Flik *et al.* 1989) and substantiating the presumed hypocalcemic nature of STC.

Next the effect of elevated plasma calcium levels on CS synthetic activity in vitro was evaluated, using quantitative immunopre-cipitation (Anderson and Blobel, 1983).

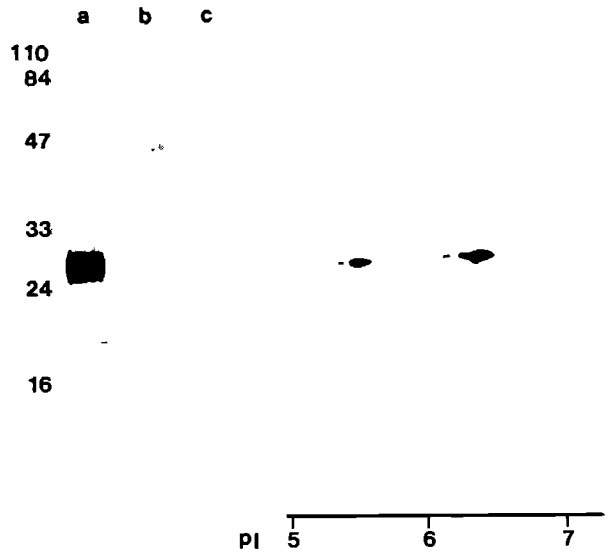

Fig. 2. *Western-blot analysis of purified stanniocalcin (STC, 1 μg). Sample treatment and presentation as in Fig. 1. Products are confined to 28-kDa region.*

CS of trout injected for 3 days with $CaCl_2$ (plasma calcium levels 4 h after the last injection were 2.67 ± 0.37 and 3.22 ± 0.25 mmol.l^{-1} for NaCl- and $CaCl_2$-injected fish, respectively; $n = 8$, $P < 0.001$) synthesize immunoprecipitable PSTC and STC at a much higher rate than that of NaCl-injected fish (Fig. 6). The radioactivity recovered from the PSTC- and STC-regions after SDS-PAGE of extract of CS of $CaCl_2$-injected fish surpassed the respective values of NaCl-injected fish by 72 and 74% (Table 1). Analysis of the radioactivity in the PSTC and STC region of the TCA-precipitate of the supernatant remaining after immunoprecipitation indicated a quantitatively successful immunoprecipitation of the newly-synthesized PSTC and STC. Addition of a surplus (10 μg per μl antiserum) trout STC hindered immuno-precipitation of newly-synthesized PSTC and STC (results not shown). Newly-synthesized PSTC and STC are released to the incubation medium (Fig. 6). Moreover, CS of $CaCl_2$-injected trout release significantly more PSTC (106%) and STC (160%) than those of NaCl-injected fish (Table 1).

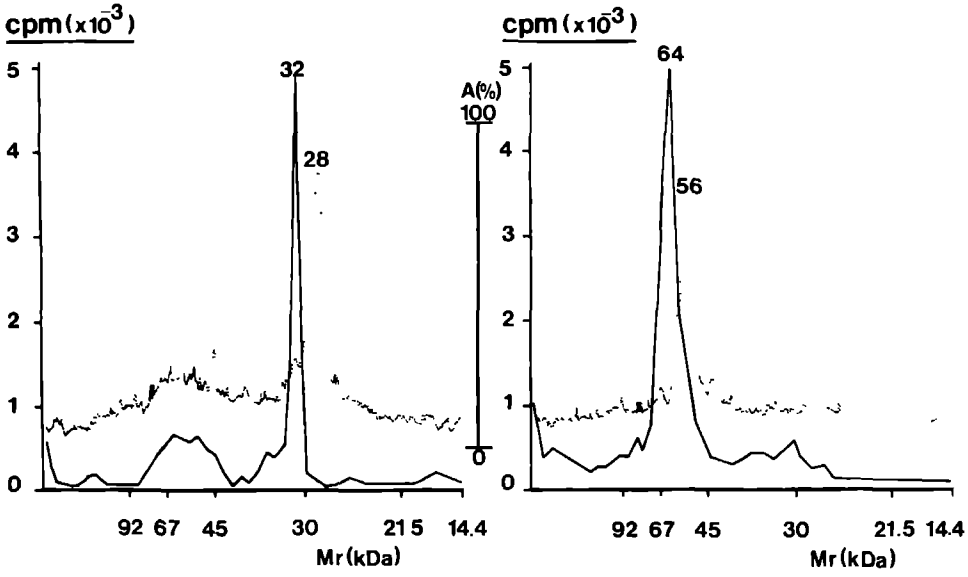

Fig. 3. *SDS-PAGE analysis of [^{35}S]-cystein labeled products formed during a 1 h pulse incubation of CS. Under reducing conditions (left panel) a 32 kDa M_r radiolabeled species is observed (solid line, sliced gel); conspicuous products stained by Coomassie Brilliant Blue (CBB) occur at the 32 and 28 kDa M_r regions (dotted line, absorption scan). Under non-reducing conditions (right panel) a 64 kDa M_r radiolabeled species and a 56 kDa CBB-stained species were observed as the predominant products. Apparent M_r values of marker proteins are given on the X-axis.*

During a 6 h incubation with [^{3}H]-glucosamine in HBSS, PSTC and STC were recovered as the major labeled species (Fig. 7). Newly-synthesized, [^{3}H]-glucosamine labeled PSTC and STC were successfully adsorbed to Con-A. The addition of 0.3 M α-methyl-D-glucoside at the start of the Con-A adsorption procedure prevented the adsorption of newly-synthesized (P)STC (results not shown). Glycopeptidase-F treatment of [^{35}S]-labeled PSTC and STC induced a 5-6 kDa shift in apparent M_r of both products. Glycopeptidase-F deglycosylated PSTC and STC were successfully immunoprecipitated by the STC antiserum RADH-I.

Two-dimensional gel electrophoresis of an extract of CS incubated for 7 h in [^{35}S]-

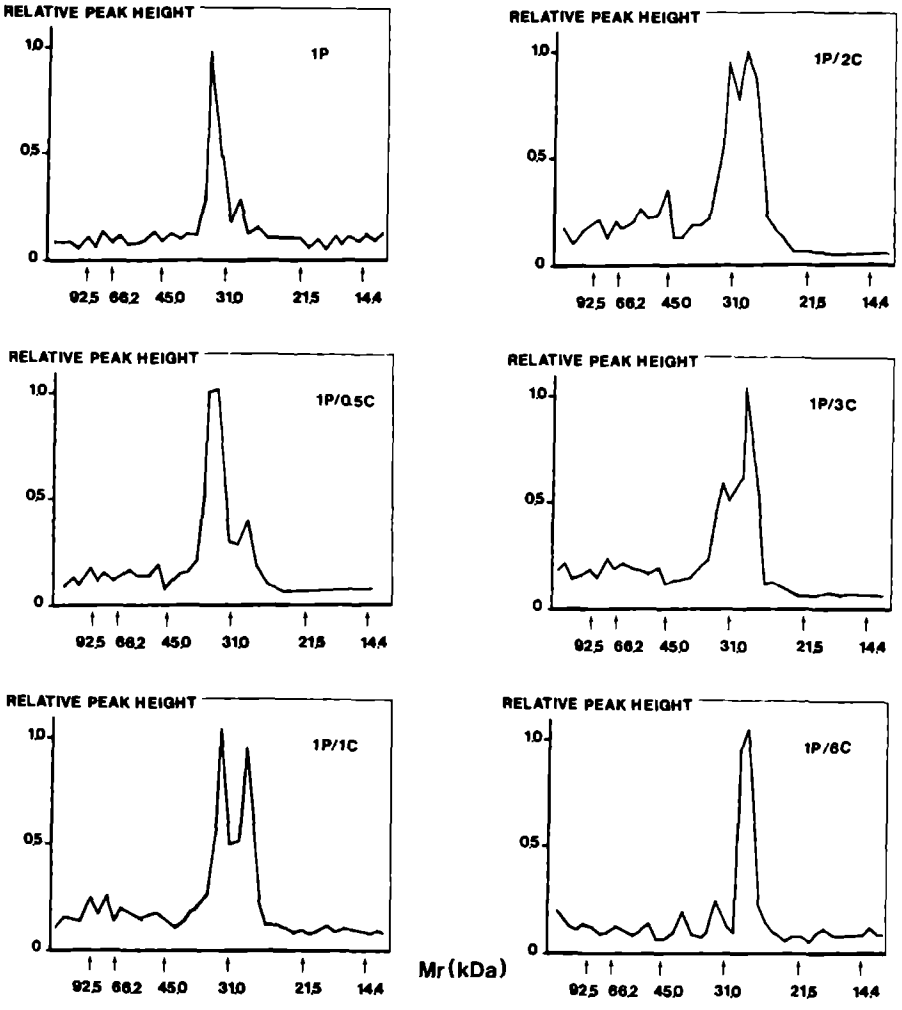

Fig. 4. *SDS-PAGE analysis (in the presence of 2-mercapto-ethanol) of CS of freshwater trout pulse labeled for 1 h in Hanks' balanced salt solution (HBSS) containing [^{35}S]-Met (1P) and subsequently chased in Medium 199 for 0.5, 1, 2, 3, and 6 h (1P/0.5C, etc.). Note the conversion of a 32 kDa M_r species into a 28 kDa M_r species. Gels were sliced (2 mm); for reasons of comparison, for each lane the slice with the highest radioactivity content was designated to represent a relative peak height 1.0, and the other slices were expressed as a fraction of this sample. Positions of marker proteins are indicated on the X-axis.*

cystein supplied HBSS revealed products in the PSTC (32 kDA) region and in the STC region (28 kDa) with pI-values between 5 and 7.2 (Fig. 8A). Immunoprecipitated, newly synthesized STC (1 h pulse in [^{35}S]-cystein in HBSS, 6 h chase) appeared as 3 major products in the 28 kDa region with pI-values around 5.0, 6.0 and 7.1; a minor 32 kDa M_r species of pI 7.0 was observed (Fig. 8B). Western blot analysis of CS extract or purified STC (Figs. 1 and 2) did not reveal products with pI values around 5 as observed for the immunoprecipitated newly-synthesized products.

DISCUSSION

Four major conclusions may be drawn from the data presented in this study. First, in line with the postulated hypocalcemic nature of STC, hypercalcemia induces release of stored STC and enhances synthesis and release of the hormone *in vitro*. Secondly, pulse-chase experiments show that trout STC synthesized *in vitro* is a 56 kDa

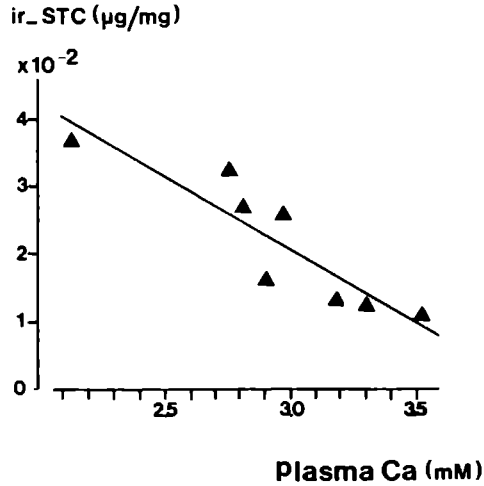

Fig. 5. *Corpuscle of Stannius (CS) immunoreactive STC (irSTC, determined by ELISA) content and plasma calcium levels in freshwater rainbow trout. The irSTC content of CS is negatively correlated with the calcemia of the fish. (r_o = -0.874, n = 8, P < 0.01). Hypercalcemia was induced by $CaCl_2$-injections (see 'MATERIAL AND METHODS').*

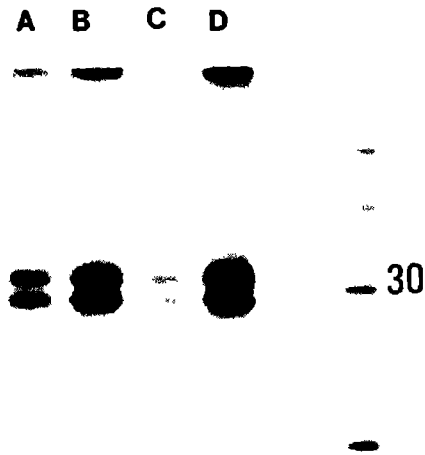

Fig. 6. *Fluorograph after SDS-PAGE analysis (reducing conditions) of immunoprecipitated products synthesized by CS of NaCl-injected (lane A) and $CaCl_2$-injected (lane B) trout, formed during a 7 h incubation with [^{35}S]-Cys in HBSS. Lanes C and D represent the immunoprecipitable products released by CS of NaCl-injected (C) or $CaCl_2$-injected (D) trout. Medium proteins were collected by trichloroacetic acid precipitation. Comparable amounts of CS wet weight were incubated. Notice the increased labeling brought about by $CaCl_2$ injections. Tissue extracts applied to the gel contained similar amounts of protein. Positions of marker proteins are indicated on the right. A quantitative analysis of this type of experiment is given in Table 1.*

glycoprotein, which is processed from a 64 kDa precursor (PSTC). In the presence of reducing agents PSTC and STC appear as a 32 and a 28 kDa M_r species, respectively, suggesting a homodimeric nature of the native molecules. Thirdly, N-linked carbohydrates make up a 4 kDa moiety of monomeric PSTC and STC. The carbohydrate moiety of STC does not contain antigenic sites for the particular antiserum used in this study. Finally, newly synthesized and immunoreactive PSTC and STC occur in several isoforms (pI's between 5 and 8), the PSTC's being slightly more acidic than the STC's. These isoforms may reflect different stages of maturation of the molecules.

Our data showing a decrease in the irSTC content of trout CS upon experimentally induced hypercalcemia, corroborate an earlier report by us on trout, goldfish and eel (Flik *et al.* 1989a) as well as reports by Lopez and coworkers (1984) who showed degranulation

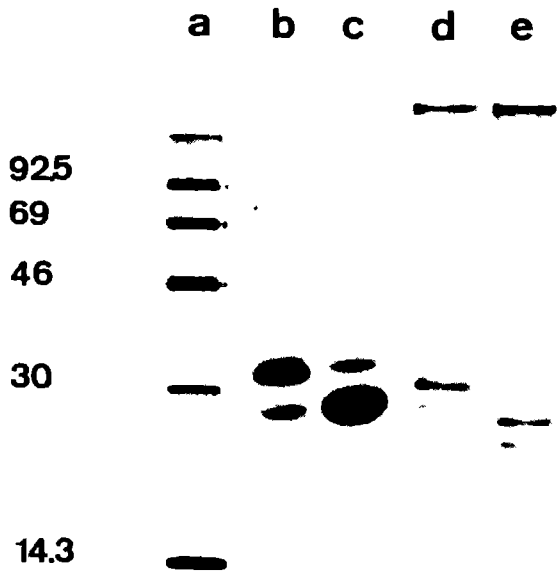

Fig. 7. *Analyses of the glycoproteinaceous nature of newly-synthesized PSTC and STC. Fluorographs after SDS-PAGE analysis (reducing conditions) of CS products labeled with [^{3}H]-glucosamine (lanes B and C) or with [^{35}S]-Cys (lanes D and E). Lanes B and C: Con-A adsorbed CS products, pulse-labeled for 1 h (B) or pulse labeled for 1 h and chased for 6 h in M-199 (C). Lanes D and E: immunoprecipitated CS products after control incubation (overnight at 37°C) for glycopeptidase-F treatment (D) and after glycopeptidase-F treatment (E). Lane A represents marker proteins; M_r-values of markers are indicated on the left.*

of the STC-cells in CS of the eel, *Anguilla anguilla* injected with $CaCl_2$, and by Lafeber and coworkers (1988a), who gave biochemical and ultrastructural evidence for release of STC by trout and eel CS after similar treatments.

Lafeber and Perry (1988) subsequently provided evidence, albeit circumstantial, that the STC released upon an experimental intra-arterial calcium challenge exerts a hypocalcemic action by inhibiting branchial Ca^{2+} influx. It is our experience that the higher dose of $CaCl_2$ used in these studies to degranulate the CS impedes the synthetic activity of the glands as measured afterwards *in vitro*. We found that applying the same protocol, but

reducing the dose by 50% (*i.e.* 0.34 mmol $CaCl_2$ per kg fish per day for 3 days) induced hypercalcemia, decreased the irSTC content of the CS and concomitantly increased biosynthetic and releasing activity of the CS *in vitro*.

The pulse and pulse-chase experiments demonstrate that a 64 and 56 kDa M_r species of trout CS have a precursor-product relationship. Most likely, the PSTC and STC molecules occur *in vivo* in this dimeric form, which appears highly susceptible to reducing agents *in vitro*. These results on newly-synthesized STC are in line with data reported by Wagner and coworkers (1986) and by Lafeber and colleagues (1988a) on STC isolated from salmon and rainbow trout: for both species the occurrence of a single N-terminal amino acid sequence of reduced STC samples was reported. Two observations further substantiate the dimeric nature of PSTC and STC. First, the incidence of a high and uneven number of half-cystines (15 on a total of 231 amino acids per monomer) in the reduced STC of Australian eel, Anguilla australis, leaves at least one cystein free for the formation of a disulphide bridge between monomers (Butkus *et al.*, 1987). Secondly, the degree of incorporation of [^{35}S]-cystein in newly-synthesized trout PSTC in its reduced and in its non-reduced form is comparable, since comparable amounts of radioactivity applied to the gel were recovered in either "64 kDa" PSTC or in "32 kDa" PSTC (see: Fig. 3). Of all amino acids tested in incorporation studies, labeled cystein consistently gave the clearest picture, being essentially confined to PSTC and STC. This observation is in line with the abundance of this amino acid in the protein core (Butkus *et al.* 1987).

The glycoproteinaceous nature of newly-synthesized (P)STC was demonstrated by the incorporation of [^{3}H]-glucosamine in the molecules and their Con-A affinity. These observations corroborate our carbohydrate analysis of isolated trout STC (Lafeber *et al.* 1988a), of which the glyco-moiety is composed of mannose, galactose and glucosamine (in the ratio 1:1:2). The STC sugar residue is sensitive to glycopeptidase-F breakdown. It must be, therefore, N-linked and may be positioned at amino acid 29. In the amino acid sequencing procedure that product released by Edman degradation from position 29 is characterized by a high polarity upon HPLC analysis, which is indicative of the presence of a sugar residue. Moreover, position 29 is followed by Ser and Thr at position 30 and 31, respectively. Linkage of N-acetyl glucosamine to aspartic acid (the most common sugar linkage) requires the sequence Asn-Xxx-Thr/Ser (Bahl and Shah, 1977) and it seems therefore very likely that a sugar residue is attached to an Asn at position 29 of the molecule. Interestingly, the Australian eel STC molecule has Asn at position 29 (Butkus *et al.* 1987).

Table. 1. *Effects of $CaCl_2$ injection in rainbow trout on synthesis and release of prostanniocalcin (PSTC) and stanniocalcin (STC) by CS in vitro. Mean values ± S.E. are given for eight tissue samples obtained in two experiments (CS were labeled for 7 h in [^{35}S]-Cys or [^{35}S]-Met). Control values for [^{35}S]-Cys labeled PSTC and STC extracted from the tissue are 48 ± 7 and 47 ± 9 Bq per mg wet weight tissue processed for SDS-PAGE, respectively, and for PSTC and STC released to the medium 12 ± 2 and 17 ± 2 Bq per mg wet weight CS incubated, respectively (n=4). Control values for tissue [^{35}S]-Met labeled PSTC and STC are 194 ± 27 and 209 ± 36 Bq per mg wet weight tissue processed, respectively, and for PSTC and STC released to the medium 46 ± 3 and 49 ± 7 Bq per mg wet weight CS incubated, respectively (n=4).* Significantly different from respective controls (NaCl), $P < 0.01$.*

Treatment	Tissue		Medium	
	NaCl	$CaCl_2$	NaCl	$CaCl_2$
PSTC, 32 kDa	100 ± 14	172 ± 17*	100 ± 13	206 ± 28*
STC, 28 kDa	100 ± 18	174 ± 24*	100 ± 13	260 ± 24*

Although sugar residues are generally highly antigenic, it appears that the polyclonal antiserum RADH-I (Kaneko *et al.* 1988) used in this study for immunoprecipitation is not directed to the sugar residue(s) of STC, as deglycosylated STC proved immunoprecipitable with the antiserum. Furthermore, the observation that both glycosylated and deglycosylated PSTC and STC reacted with RADH-I seems to exclude the possibility that the N-terminals of (P)STC are recognized by this antiserum.

The 5 to 6 kDa shift in apparent M_r upon deglycosylation of the reduced PSTC and STC is consistent with a 12% (6.5 kDa) carbohydrate content of an isolated trout STC (Lafeber *et al.* 1988a). We, therefore, conclude that only N-linked sugar residues are present in (P)STC. Experiments with the enzyme endo-α-N-acetylgalactosaminidase (EC3.2.1.97),that splits off O-linked sugar residues did not change the apparent M_r of (P)STC (data not shown) and this further substantiates that O-linked glyco-moieties are absent. Butkus and colleagues (1987) reported a comparable 4 kDa shift in apparent M_r upon deglycosylation of Australian eel monomeric STC. This 4 kDa glyco-

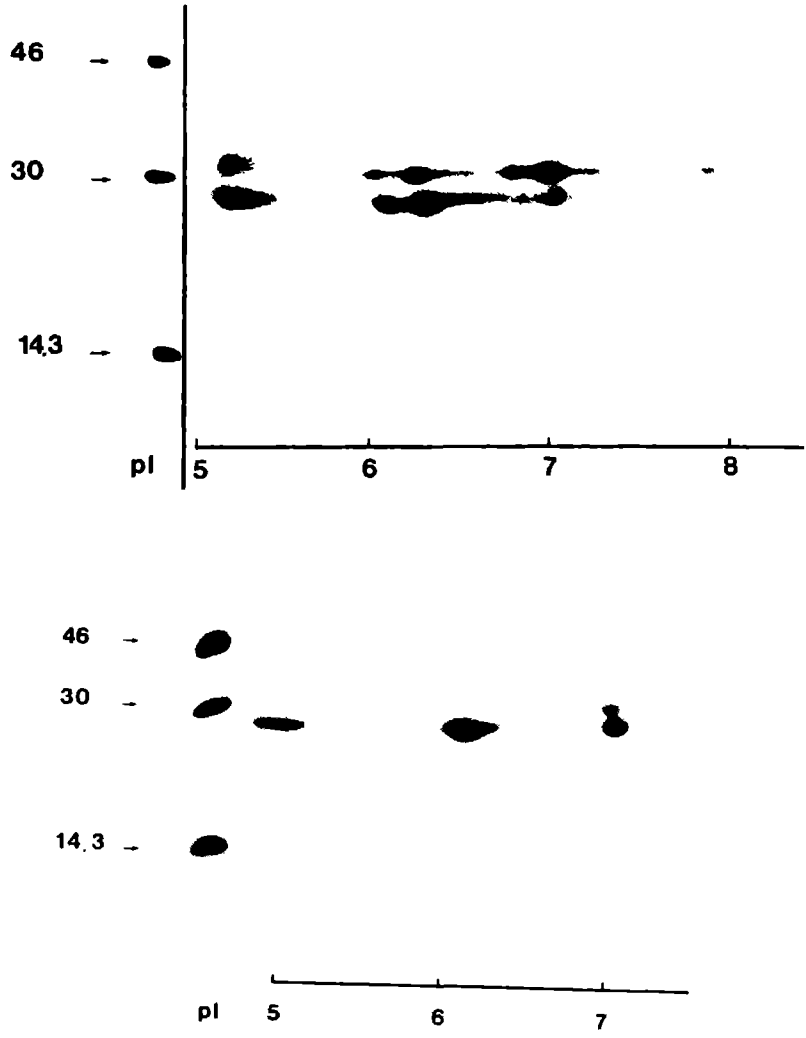

Fig. 8. *Fluorograph after two-dimensional gel electrophoretic analysis (reducing conditions) of CS products labeled with [^{35}S]-Cys during a 7 h incubation (A) and immunoprecipitated products formed during a 1 h pulse labeling followed by a 6 h chase period (B). M_r values of marker proteins are given on the Y-axis, pI values of marker proteins are given on the X-axis.*

moiety made up the difference between the native glycosylated monomer and the predicted protein core of the molecule.

The occurrence of several isoforms of PSTC and STC revealed by two-dimensional gel electrophoresis does not imply, in our opinion, that the CS produce different PSTC and STC molecules. The prosequence of Australian eel PSTC (Butkus *et al.* 1987) contains two basic Arg's and 3 acid aminoacids (2 Glu and 1 Asp), giving it a net acid nature. Assuming the same composition for the prosequence of trout STC, it could be the amino acid composition of the prosequence that determines this shift. However, also acetylation (Smyth *et al.* 1979), deamination (Rudman *et al.* 1979) and the process of maturation of the glyco-residues (Crine *et al.* 1979; Loh, 1979) may produce different glycoprotein species and such phenomena as well might underlie the isoforms of PSTC and STC observed. The demonstration of immunoprecipitated, newly-synthesized products with pI values around 5 and the absence of products with pI values around 5 in Western blots of CS extracts or purified STC, suggests to our opinion that very low levels of STC isoforms are present in the cell during hormone processing that are only detected by radiolabeling techniques.

CHAPTER 4

Regulation of secretion of the teleost fish hormone stanniocalcin: effects of extracellular calcium

SUMMARY

The release *in vivo* and *in vitro* of stanniocalcin (STC) from the corpuscles of Stannius (CS) of the rainbow trout and the European eel was studied. Intraperitoneal injection of $CaCl_2$ (2.45 mmol.kg^{-1} fish) leads to an elevation of both ionic and total calcium in the plasma and results in the release of STC from the CS into the blood. Release of STC *in vitro* is not affected at 'physiological' (1.0-1.5 mM) or lower Ca^{2+} levels in the incubation medium. High levels of Ca^{2+} (2.5 mM and higher) however stimulate the release of STC, in particular that of stored STC. We hypothesize that variations in extracellular Ca^{2+} in the normocalcemic range do not directly regulate STC-release.

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INTRODUCTION

The predominant calcitropic hormone in teleost fish is stanniocalcin (STC), previously referred to as hypocalcin (Pang *et al.* 1974) or teleocalcin (Ma and Copp, 1978). It is produced by the corpuscles of Stannius (CS)(Milet *et al.* 1979b; Fenwick, 1982; Wagner *et al.* 1986; Wendelaar Bonga and Pang, 1986; Flik *et al.* 1989a). We have shown that in European eels this hypocalcemic hormone exerts its function by controlling plasma ionic calcium (Hanssen *et al.* 1989).

In mammals the secretion of two major calcitropic hormones is probably regulated directly by the extracellular calcium level. The parathyroid gland parathormone (PTH) secretion is sigmoidally related with extracellular ionic calcium (Brown, 1983). Increased calcitonin secretion by C-cells is correlated with elevated serum calcium levels (Austin *et al.* 1979); this has led to the conclusion that external calcium is involved in the regulation of calcitonin secretion.

The role of extracellular Ca^{2+} as a regulator of the secretory activity of the CS is hitherto unclear. Lopez *et al.* (1984) showed that experimentally induced hypercalcemia in the European eel leads to a complete hormone depletion from the CS cells. $CaCl_2$-infusions in rainbow trout caused a degranulation of CS cells accompanied by acute inhibition of whole body Ca^{2+} influx (Lafeber and Perry, 1988), suggesting a rapid and direct effect of elevated plasma Ca^{2+} on the release of STC. Flik *et al.* (1989a) reported that in goldfish, trout and eel, $CaCl_2$ injections induced a rise in plasma calcium and a release of STC from the CS. A direct effect of extracellular calcium on the CS cells was suggested before by the results of *in vitro* experiments: coho salmon CS showed enhanced exocytosis when incubated in high Ca^{2+} media but not in low Ca^{2+} media (Aida *et al.* 1980). STC release by rainbow trout CS cells in primary culture is dose- dependently stimulated by extracellular calcium (Wagner *et al.* 1989).

In this study we investigated whether the experimentally induced and acutely stimulating effect of a rise in extracellular calcium on the secretory activity of the CS indeed plays a role in the *in vivo* control of STC secretion. To obtain well defined extracellular Ca^{2+} levels and to exclude other factors affecting *in vivo* release of STC (blood-borne factors, innervation) we studied the effect of extracellular Ca^{2+} on STC release *in vitro*. The effects of Ca^{2+} concentrations below, above, and in, what is assumed, the physiological range (1.0-1.5 mM) were examined on the release *in vitro* of total immunoreactive (stored) and newly synthesized STC. Also the effect of

calimycin A23187, which increases the intracellular Ca^{2+} concentration (Foreman *et al.* 1973), was tested on STC release. Levels of immunoreactive STC were assessed with an ELISA technique.

MATERIAL AND METHODS

Animals

European eels (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*), weighing around 250 g, were kept in 1000 l tanks supplied with running Nijmegen tapwater (main ion concentrations in mM :Ca^{2+}, 0.7; Na^{+}, 1.9; Cl^{-}, 3.1; Mg^{2+} 0.2; temperature 12 °C). Rainbow trout were fed daily with Trouvit pellets. The eels were not feeding. The animals had been acclimated to laboratory conditions for at least 14 days. Freshly dissected CS of eel and trout were carefully freed of connective tissue and kidney tissue and collected in Hanks' balanced salt solution (HBSS; Flow laboratories).

$CaCl_2$ injections and plasma analysis

Rainbow trout were injected intraperitoneally with 300 μl of a $CaCl_2$ solution (2.04 M $CaCl_2$ in 0.9% NaCl), a treatment previously shown to produce a significant hypercalcemia (Flik *et al.* 1989). Control fish were injected with 300 μl 0.9% NaCl. Four hours after injection a bloodsample was taken by puncture of the caudal vessels. Fish were killed by spinal transsection and the CS were removed. CS were homogenized in 250 μl 0.1 M acetic acid. Blood samples were analyzed as described before (Hanssen *et al.* 1989). Blood ionic calcium was measured with an ionic calcium analyzer (ICA-1, Radiometer). Plasma total calcium and protein concentrations were determined with commercial reagent kits (Sigma and Biorad, respectively). Bovine serum albumin (Biorad) was used as a protein reference.

Incubations

Incubation media were prepared by adding $CaCl_2$ to Ca^{2+}-free HBSS (pH 7.4). In media designated '0 mM Ca^{2+}' no $CaCl_2$ was added. The addition of 1 mM EGTA to a 0.65 mM Ca^{2+} medium resulted in a final Ca^{2+} concentration of 0.1 μM (Sillen and Martell, 1964; Van Heeswijk *et al.* 1984). Calimycin A23187 was added from a stock solution (5 mg.ml^{-1} in DMSO/methanol 1:9). In control media the solvent was included and it never exceeded 0.1% v/v). Per incubation two corpuscles, each from different eels or from different trout, were transferred to an incubation vessel containing 50 μl incubation medium supplemented with 925 KBq ^{3}H-leucine (Amersham, specific activity 5,2 TBq/mmol). Incubation was for 3 hours at 28 °C. Next the CS were washed in 2 ml incubation medium (3 times) and incubated for another 3 hours in 200 μl medium. Since ^{3}H-STC was not only synthesized but also released during the first incubation period, it was necessary to carry out a second incubation without radiolabel, in which the release of ^{3}H-STC was studied. Incubation media were collected and the CS were homogenized in 250 μl 0.1 M acetic acid. Part of the incubation media and the CS homogenate were used for ELISA. The remainder of the media was precipitated with 10% TCA (4°C overnight), and the remainder of the CS homogenate was lyophilized. Radioactivities in media and CS homogenates were determined in a LKB Rackbeta LSA with a dpm-program. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli,1970) of precipitated proteins from incubation media and CS homogenates indicated that at least 90% of the labeled product (based on optical scans from fluorographs) was a 28 kD (trout) or 30 kD (eel) product that could be identified as trout or eel STC, respectively (Flik *et al.* 1989).

Enzyme-linked immunosorbent assay (ELISA)

A non-competitive ELISA was carried out according to the method of Kaneko *et al.* (1988), with some modifications. The STC antiserum used was RADH-1 (Kaneko *et al.* 1988) raised against trout STC. Eel STC showed a high degree of cross reactivity with the RADH-1 antiserum (Flik *et al.* 1989). Serial dilutions of the antigen were tested for the presence of STC. Trout and eel CS homogenate (prepared as described before), trout and eel CS incubation media, and trout plasma produced dose-response curves parallel to the trout STC standard. The wells of microtiter plates (Nunc,

immunoplate maxisorp type 1) were precoated for 2 hours at 20 °C with 100 μl 1% glutaraldehyde. Wells were washed with distilled water and coated with 100 μl serial dilutions of STC or with the unknown samples in coating buffer (0.1 M sodium bicarbonate buffer, pH 9.4, with 0.05% Triton X-100; Biorad). Antigen binding was for 1 hour at 37 °C and subsequently 16 hours at 4°C. Between incubation steps wells were washed with buffer (0.02 M sodium phosphate-buffered saline; PBS, pH 7.4, with 0.05% Tween-20; Biorad). The remaining binding sites in the wells were blocked with 100 μl block buffer (2% bovine serum albumin in PBS) for 1 hour at 37 °C. Wells were washed and incubated with 100 μl 1:10.000 RADH-1 in block buffer for 90 min. at 37 °C. Control wells were incubated with block buffer only. Wells were washed and incubated with 100 μl 1:2000 goat-anti-rabbit peroxidase immunoconjugate (Nordic) in block buffer, for 1 hour at 37 °C. The amount of peroxidase immunoconjugate bound to the wells was quantified using o-phenylene diamine (OPD; Sigma) as a substrate. After washing the wells, 250 μl substrate (0.1% OPD in 0.1 M Na_2HPO_4, 0.1 M citric acid, 0.05% H_2O_2, pH 5.2) was added at 20 °C. The peroxidase reaction was stopped after 2 min. with 100 μl 4 M H_2SO_4. Absorbance was measured at 492 nm using a EAR-400 microplate reader (SLT lab instruments).

Electron microscopy

Eel CS, incubated as described above were fixed for 10 min. in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), at 20 °C. They were then postfixed in a solution containing 0.66% osmium tetroxide, 1% glutaraldehyde and 1.66% potassium bichromate, in sodium cacodylate buffer (pH 7.4), for 1 hour at 4 °C. The CS were block-stained in 2% aqueous uranyl acetate. The tissues were dehydrated and embedded in Spurr's resin. Ultrathin sections were poststained with lead citrate and examined in a Philips EM 201 electron microscope.

Calculations and statistics

The STC contents of CS of fishes of similar weight showed a high degree of variability. Therefore, the results of *in vitro* experiments have been presented as percentages STC released into the incubation medium. Data are presented as means $\pm$ S.E.M. For statistical evaluation the Mann-Whitney *U*-test was used. Significance was accepted at $P < 0.05$.

RESULTS

In vivo studies

The effects of a rise in blood calcium concentration on STC release are shown in Fig. 1A,B. Following $CaCl_2$ injection blood ionic and total calcium levels are elevated from 1.25 to 3.75 mM and from 2.50 to 5.75 mM, respectively (Fig. 1A). Concomitantly, the plasma immunoreactive STC level had increased from 175 to 800 $ng.ml^{-1}$ and the CS-STC content had decreased from 600 to 325 $ng.\mu g^{-1}$ protein, as determined by ELISA (Fig. 1B).

In vitro studies

A comparison between the release of newly synthesized and total immunoreactive STC from trout and eel CS at a medium Ca^{2+} concentration of 1.25 mM is shown in Fig. 2. The release of total immunoreactive STC from trout and eel CS during the labeling incubation period and the non-radioactive incubation period thereafter, was similar. In trout CS 70% of the newly synthesized STC was released during the 3 hour labeling period and only 4% during the following 3 hour incubation period. For eel CS these figures were 40% and 16%, respectively. Because of the relatively high basal release of newly synthesized STC during the non-radioactive incubation period we selected eel CS for investigation of the effects of medium Ca^{2+} levels on CS secretory activity. *In vitro* incubation of eel CS showed that the release of newly synthesized and total immunoreactive STC was not significantly affected by medium Ca^{2+} levels ranging from 0 to 2 mM (Fig. 3). EGTA added to obtain incubation media with a final Ca^{2+}

concentration of 0.1 μM did not diminish the secretory response. Increased release of newly synthesized and total immunoreactive STC was observed at 2.5 and 3.75 mM Ca^{2+}. Examination of electron micrographs of eel CS following incubation under various conditions (Fig. 4 A-D) showed marked degranulation at 3.75 mM; at '0'mM, 0.1 μM and 1.25 mM Ca^{2+} no degranulation was observed. The effects of calimycin (A23187) have been summarized in Fig. 5. Calimycin at a concentration of 1.3×10^{-5} M increases release of both newly synthesized and total immunoreactive STC.

DISCUSSION

The non-competitive ELISA described here appears suitable for the quantitation of STC. Both trout and eel STC could be easily detected with a sensitivity of approximately 18.5 pM (the M_r for trout STC is 54 kDa; Flik *et al.* 1989a). The STC-ELISA and the STC-RIA used by Kaneko *et al.* (1988) have a sensitivity of approximately 74 pM and 18.5 pM, respectively. The STC-RIA developed by Wagner *et al.* (1989) has a detection limit of approximately 74 pM.

Our results clearly show that experimentally induced high extracellular Ca^{2+} levels evoke an increased release of STC *in vivo* as well as *in vitro*. Assuming that such high levels of extracellular Ca^{2+} do not occur under natural conditions, we conclude that plasma Ca^{2+} is normally not a determining factor in the direct control of STC release.

In vivo

In the present study, hypercalcemia induced by $CaCl_2$ injection resulted in the release of immunoreactive STC from the CS. This observation confirms reports showing that hypercalcemia induced by $CaCl_2$ injection causes depletion of the hormonal content of CS cells (Lopez *et al.* 1984; Lafeber and Perry, 1988; Flik *et al.* 1989a). These findings are consistent with the assumed hypocalcemic function of CS in fish (Pang *et al.* 1974; Milet *et al.* 1979b; Fenwick, 1982; Wagner *et al.* 1986; Wendelaar Bonga and Pang, 1986). With a mean CS weight of 2.5 mg per fish we calculated that, following the $CaCl_2$ challenge, about 86 μg STC was released into the

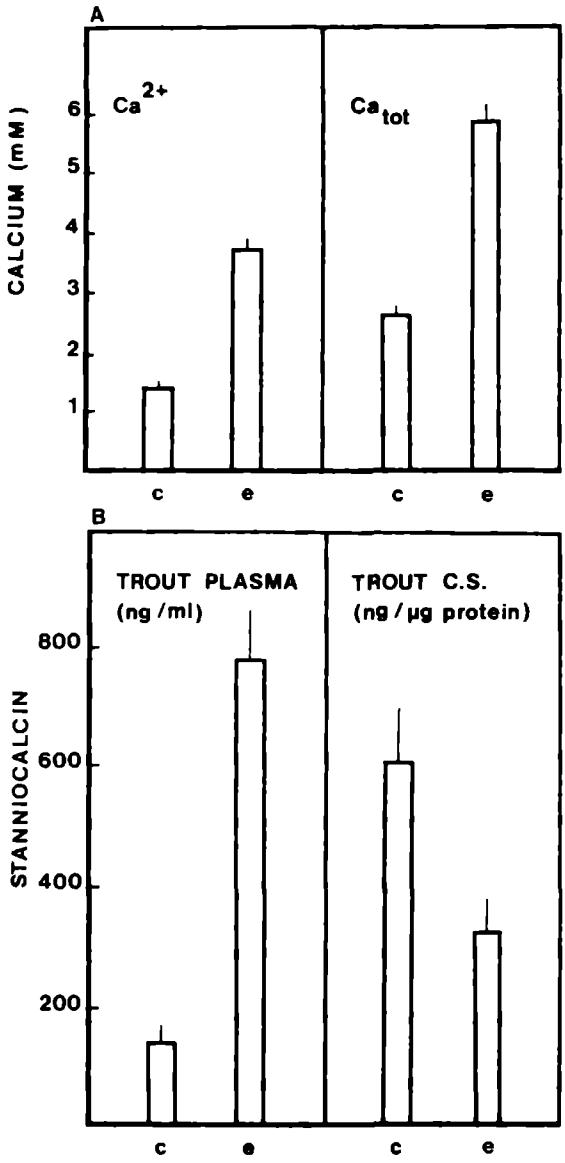

Fig. 1 . *Effect of $CaCl_2$ injection (e) on ionic and total plasma calcium concentrations (Fig. 1A) and on the STC concentrations in plasma and CS homogenate (Fig. 1B) in rainbow trout. Saline injections served as control (c). (N = 8).*

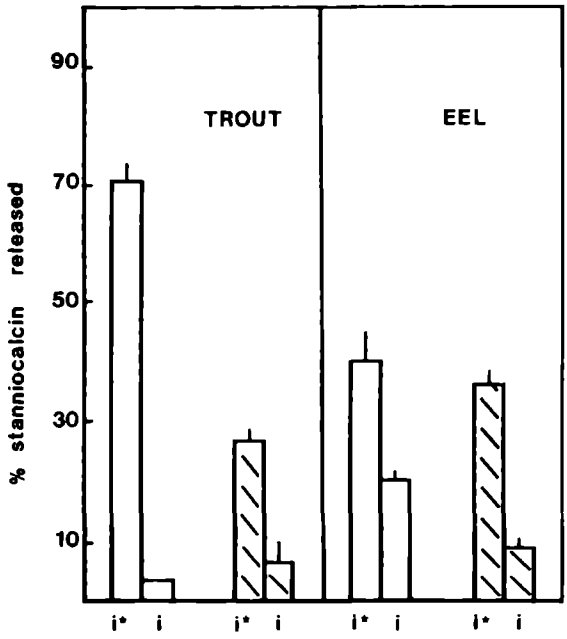

Fig. 2. *Release of newly synthesized (□) and total immunoreactive (▨) STC from trout and eel CS in 1.25mM Ca^{2+} medium. STC in labeling incubation medium (i*), non-radioactive incubation medium (i), and CS homogenate together is 100%. (N = 4).*

blood (1 mg CS contained about 125 μg extractable protein). Our ELISA data show a rise in plasma immunoreactive STC after $CaCl_2$ injection from 175 ng.ml^{-1} to 800 ng.ml^{-1}, equivalent to a rise from 3.2 to 14.8 nM, respectively. Assuming a plasma volume of 7.5 ml (3% of the body weight) the total increase in STC will amount to 4.7 μg, at four hours after injection, indicating that 95% of the STC initially released has been cleared from the plasma.

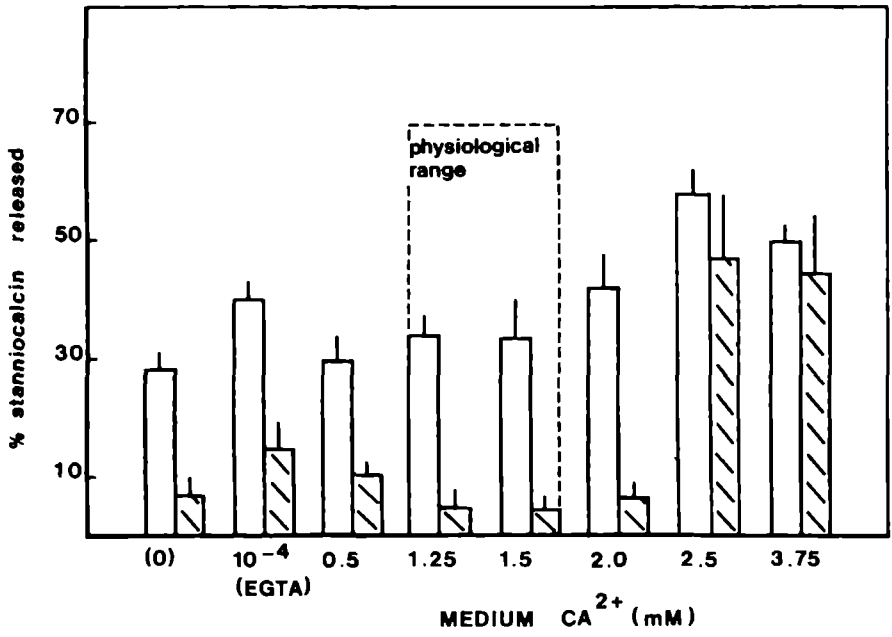

Fig. 3. *Effect of the medium Ca^{2+} concentration on the release of newly synthesized (□) and total immunoreactive (▨) STC from eel CS. STC in non-radioactive incubation medium and CS homogenate together is 100% (N = 8).*

In vitro

We found that variations in extracellular Ca^{2+} *in vitro* between 0 and 2.0 mM did not significantly affect the basal release of newly synthesized or total immunoreactive STC. Application of the Ca^{2+}-chelator EGTA, which in our experimental design reduced external Ca^{2+} to 0.1 μM (Sillen and Martell, 1964; Van Heeswijk *et al.*, 1984), did not decrease basal STC-release. Electron micrographs of CS incubated in '0' mM, 0.1 μM and 1.25 mM Ca^{2+} medium all showed a large number of secretory granules and no apparent signs of altered secretory activity. However, incubation of CS in 2.5 or 3.75 mM Ca^{2+} media stimulated release of both newly synthesized and total

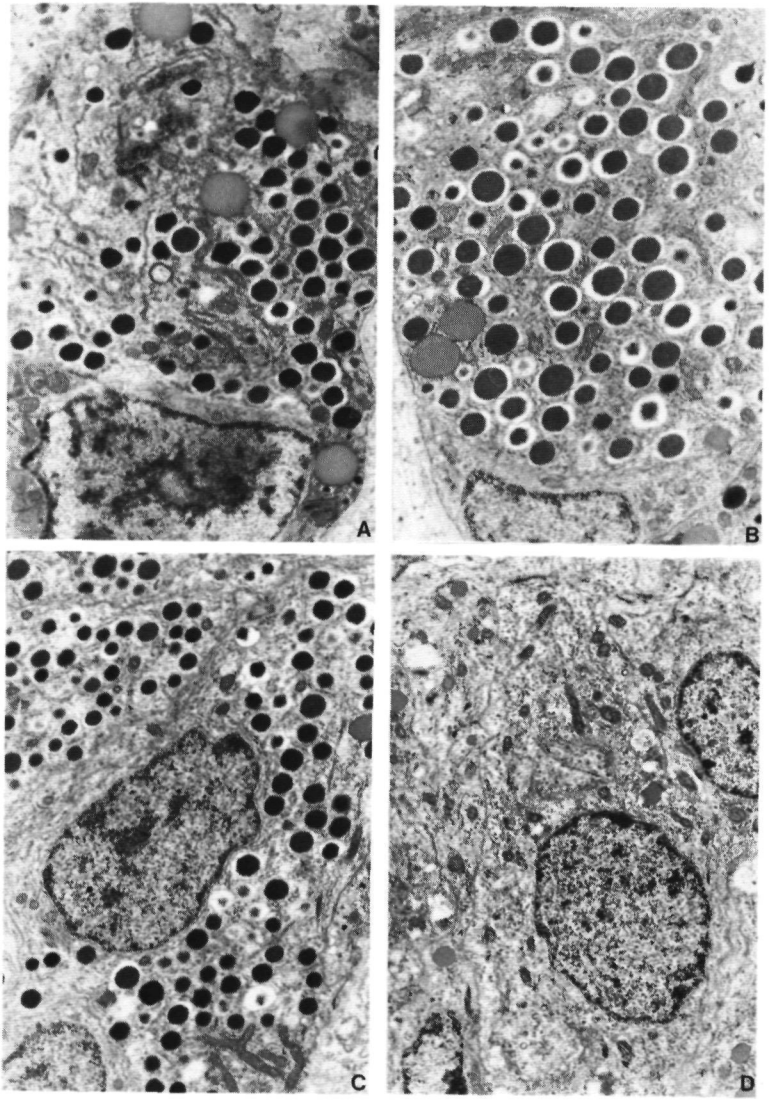

Fig. 4. *Electron micrographs of eel CS after incubation in media containing 0.1 μM (4A), '0' mM (4B), and 1.25 mM (4C)* Ca^{2+} *show CS cells with many secretory granules. Incubation in 3.75 mM* Ca^{2+} *(4D) leads to degranulated cells. Magnification, 15,000x.*

immunoreactive STC, and electron micrographs of CS incubated in 3.75 mM Ca^{2+} medium showed marked degranulation of the cells.

A stimulatory effect on the newly synthesized and total immunoreactive STC-release was also obtained with calimycin (A23187). The equipotent stimulatory effect of high external Ca^{2+} (2.5-3.75 mM) and calimycin (1.3×10^{-5} M) on STC-release indicates that STC release may be triggered by a surge of internal Ca^{2+}. Since addition of $CoCl_2$ to the medium inhibits high external Ca^{2+}-stimulated STC secretion (Wagner *et al.* 1989), STC-release is probably triggered by a Ca^{2+}-influx through voltage-independent Ca^{2+} channels in the plasma membrane. External Ca^{2+} concentrations in the physiological range (1.0-1.5 mM) or lower, are apparently unable to open these channels, since they do not induce release. With respect to high external Ca^{2+} levels our results are in line with histological observations of Aida *et al.* (1980), who found degranulated cells in salmon CS tissue after incubation in media containing 3 or 6 mM Ca^{2+} and after treatment with calimycin (A23187) in the presence of 1.5 mM Ca^{2+}. These authors also found that media containing 1.5 mM Ca^{2+} or lower did not induce degranulation. After addition of EDTA resulting in a Ca^{2+} concentration of 0.16-0.21 mM (values that we calculated from their data) no stimulatory effect on degranulation occurred. However, Wagner *et al.*(1989) showed a dose-dependent stimulation of the STC-release from trout CS cells in primary culture between 1 and 2.5 mM external Ca^{2+} (above 2.5 mM, release did not increase any further). In accordance with our results, these authors found that calimycin mimicked the stimulatory effect of 1.8 mM external Ca^{2+} on STC-release. Wagner *et al.* (1989) found no stimulation of the STC-release after lowering the medium Ca^{2+} concentration to 0.8 mM or 1 μM (values that we calculated from their data), by addition of 1 or 2 mM EGTA to 1.8 mM Ca^{2+} medium, respectively. The enhanced STC release at increasing Ca^{2+} concentrations between 1 and 2.5 mM found by Wagner *et al.* (1989) is not consistent with our results. This difference may be the result of species- specific differences or of the way of preparation of the primary CS cell suspension, which inevitably affects the cellular membrane and possibly cellular Ca^{2+}-homeostasis.

In our experiments a basal STC-release was found at external Ca^{2+} concentrations around and below the physiological range which is not accompanied by obvious degranulation of the CS cells. Even at an external Ca^{2+} concentration of 0.1 μM the basal release has not changed. This indicates that at low external Ca^{2+} concentrations, a Ca^{2+}-influx from the medium does not occur or is insufficient to initiate STC release. The basal STC release may depend on Ca^{2+} release from intracellular stores. However,

one may not exclude the possibility of a low rate of secretion at basal or even reduced levels of intracellular Ca^{2+} (Penner and Neher, 1989).

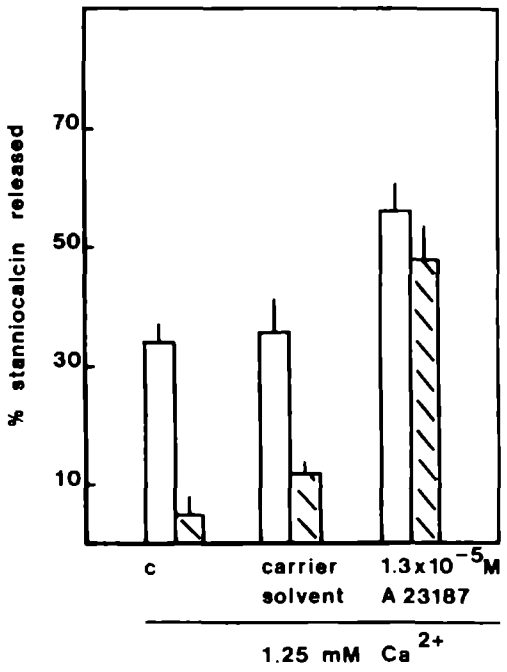

Fig. 5. *Effect of 1.3×10^{-5} M calimycin (A23187) added to 1.25 mM Ca^{2+} medium on the release of newly synthesized (□) and total immunoreactive (▨) STC from eel CS. Media containing 1.25 mM Ca^{2+} and 1.25 mM Ca^{2+} with an equivalent amount of carrier solvent were used as controls. STC in non-radioactive incubation medium and CS homogenate together is 100%. (N = 8)*

secretory pools

When CS are incubated in media with 2.5 or 3.75 mM Ca^{2+}, the release of total STC has increased 7 times and that of newly synthesized STC only 1.7 times. This suggests that upon stimulation by high external Ca^{2+}, stored STC is preferentially released. This is in accordance with the electron micrographs of cells from CS incubated in 3.75 mM external Ca^{2+} medium.

We conclude that there are more STC pools in the CS cells which are controlled separately. One pool is characterized by the presence of newly synthesized STC that appears to be secreted independently of an external stimulus; another pool contains stored STC that is released upon stimulation by factors that induce increased Ca^{2+}-influx in the CS cells. Separate intracellular hormone pools, one consisting of newly synthesized hormone, the other consisting of stored product, have been described in a variety of endocrine cells (*e.g.* Morrisey *et al.* 1979). Experiments by Walker and Farquhar (1980) have shown the existence of two individually recruitable secretory pools in prolactin cells of the rat. Newly synthesized prolactin was preferentially released in unstimulated cells whereas TRH-stimulated prolactin cells preferentially release stored prolactin. These observations parallel our results on STC release.

Physiological significance

Several authors have suggested that the secretory activity of the CS is directly controlled by variations in external (plasma) Ca^{2+} levels (Aida *et al.* 1980; Flik *et al.* 1989a). However, the high Ca^{2+}-stimulated STC release *in vivo* does not allow this conclusion: the hypercalcemia induced by $CaCl_2$ injection in our studies as well as in the studies cited above (3.75 mM or higher; Lopez *et al.* 1984; Lafeber and Perry, 1988; Flik *et al.* 1989a) is above the assumed physiological range (1.0 - 1.5 mM). Our *in vitro* data show that differences in Ca^{2+} concentrations in a range corresponding to physiologically relevant plasma Ca^{2+} concentrations have no effect on STC secretion. We conclude therefore, that the high Ca^{2+} stimulus for the in vitro release of stored STC most likely has no physiological significance since its concentration is supra physiological. We suggest that, at least in the eels, effects of plasma Ca^{2+} variations on STC secretion *in vivo* are indirect and possibly mediated by the nervous system. The CS are richly innervated (Krishnamurthy, 1971; Wendelaar Bonga *et al.* 1977) and

observations by Unsicker *et al.* (1977) demonstrated the presence of nerve fibers containing noradrenalin, adrenalin, and 5-hydroxy-tryptamine in the CS of *Salmo irideus*. Preliminary results show a stimulatory effect of the acetylcholine agonist carbachol on the STC release. This indicates that a neural factor should be considered to be involved in the regulation of STC secretion in the eel and trout as well.

CHAPTER 5

Influence of the ambient calcium on stanniocalcin secretion in the european eel (*Anguilla anguilla*)

SUMMARY

The cells of the corpuscles of Stannius appeared to be more active in eels acclimated to sea water than in eels acclimated to fresh water. In acclimated eels however, total and ionic plasma calcium concentrations and stanniocalcin titers did not differ. This suggests that levels of stanniocalcin in freshwater acclimated eels are sufficient to maintain normocalcemia in sea water. When freshwater acclimated eels were transferred directly to sea water, total and ionic calcium concentrations in the plasma increased significantly within 24 h but there was no apparent effect on the corpuscles of Stannius within the same time frame. This suggests that changes in secretory activity of the corpuscles of Stannius do not occur rapidly when they are presented with a hypercalcemic challenge. Conversely, when seawater acclimated eels were transferred to fresh or distilled water there appeared to be a very rapid reduction in secretory activity of the corpuscles of Stannius as indicated by a rapid accumulation of secretory granules. These data suggest that stanniocalcin turnover is more rapid in seawater acclimated eels and that the secretory activity of the corpuscles of Stannius is rapidly reduced when a hypercalcic challenge is removed.

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J. Exp. Biol. (1991b; in press)

INTRODUCTION

Euryhaline fish, like the eel, face extensive changes in environmental calcium concentrations upon migration between fresh water and sea water. In fresh water the ionic calcium level can be as low as one-tenth of that of the blood, whereas in sea water the ionic calcium level may reach values 10 times higher than that of the blood. Since euryhaline fish maintain their blood calcium levels quite precisely in both fresh and sea water (Chan and Chester Jones, 1968), they must be able to regulate their blood calcium in accordance with the environmental calcium challenges associated with migration.

Of all the fish endocrine systems studied the corpuscles of Stannius (CS) have been most consistently implicated in the control of plasma calcium metabolism (Wendelaar Bonga and Pang, 1991). Stanniocalcin (STC), the hormone secreted by the CS, lowers plasma calcium levels by reducing gill calcium uptake (Fenwick and So, 1974; Milet *et al.* 1979a; So and Fenwick, 1979; Lafeber and Perry, 1988; Lafeber *et al.* 1988c). Histological examination of the CS has revealed that the CS are consistently more active in seawater-adapted fish than in freshwater-adapted fish (Olivereau, 1964; Hanke *et al.* 1967; Wendelaar Bonga *et al.* 1976; Meats *et al.* 1978). In addition, CS of fish kept in calcium-deficient sea water appear to be inactive (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980), suggesting that glandular activity is directly related to environmental calcium concentration. Such observations have led to the suggestion that the release of STC is controlled directly by changes in plasma calcium levels (Aida *et al.* 1980; Wagner *et al.* 1989). This hypothesis was supported by observations on the effects of exposure of cells from the corpuscles of Stannius *in vitro* to media of various calcium concentrations on STC secretion. However, the rise in the extracellular calcium level required to stimulate STC release *in vitro* is outside the physiological range ($[Ca^{2+}] > 2$ mM; Hanssen *et al.* 1991a).

We have studied the regulation of STC release by examining CS from eels exposed to changes in the ambient water calcium concentration. The effects of rapid transfers between fresh water and sea water on the ultrastructure of the CS and on the plasma calcium and STC concentrations were examined.

MATERIALS AND METHODS

Animals

Sexually immature freshwater European eels (*Anguilla anguilla* L.), weighing between 100 and 300 g, were obtained from a commercial fish dealer in the Camarque, France. Fish were kept in well aerated 1000 l tanks at approximately 12 °C. Eels were acclimated to circulating city of Nice tap water (1.63 mM Ca^{2+}, 0.12 mM Na^{+}, 0.09 mM Cl^{-}) or Mediterranean sea water (12.50 mM Ca^{2+}, 530 mM Na^{+}, 552 mM Cl^{-}) for at least 3 weeks. The eels were not fed.

Experimental protocol

Freshwater-acclimated eels were netted and transferred directly to 200 l tanks containing circulating fresh water or sea water for 24 h. Seawater-acclimated eels were transferred for a 24 h period to seawater tanks, freshwater tanks or tanks containing running distilled water (Ca^{2+} not detectable with a commercial calcium kit, pH 7.0). At the end of the experiment fish were anaesthetized in ethylaminobenzoate (MS222, 2.5 $g.l^{-1}$, pH 7.8) and blood samples were taken by puncture of the caudal vessels. The CS were removed as described before (Hanssen *et al.*, 1989) and prepared for electron microscopy.

Plasma analysis

Plasma ionic and total calcium concentrations were measured as previously described (Hanssen *et al.* 1989). Plasma ionic calcium was determined with an ionic calcium analyzer (ICA-1, Radiometer). Total plasma calcium was measured with a commercial calcium reagent kit (Sigma).

Enzyme-linked immunosorbent assay (ELISA)

A competitive ELISA technique, developed and validated by Mayer-Gostan *et al.* (1991), was used to quantitate STC levels in plasma samples. This technique is based on competition between free STC in standard or plasma samples and STC immobilized on microtiter plates for the STC antibodies. The trout STC antiserum used (RADH-1; Kaneko *et al.* 1988), has a high degree of crossreactivity with eel STC. Purified trout STC (Lafeber *et al.* 1988a) served as a standard.

The wells of microtiter plates were coated with trout STC (1.85 nM) in 200 μl of coating buffer (0.05 M sodium bicarbonate buffer, pH 9.6), except for the wells in the first column of the plate which received 200 μl of a BSA solution of equivalent protein content in coating buffer (blanks). Coating lasted for 1 h at 37 °C; coated plates were stored at 4 °C. Between incubation steps, coated plates were rinsed with washing buffer (0.01 M sodium phosphate buffered saline; PBS, pH 7.4, with 0.05 % Tween-20; Bio-Rad). For competition, antigen and RADH-1 antiserum were diluted in dilution buffer (*i.e.* washing buffer containing 2% porc serum). Equal volumes of antigen and diluted antiserum (1 : 80,000) were incubated in 4 ml Minisorp tubes (Nunc) for 16 hours at 20 °C. Tubes containing diluted antiserum only, were incubated under the same conditions. After washing, the wells of coated plates were blocked with 200 μl of dilution buffer (containing 2% porc serum) for 1 h at 37 °C. The plates were washed and filled with 200 μl per well of the antigen/antiserum mixture except for the wells in columns 1 (blanks) and 2 (B_0) which received 200 μl of the diluted antiserum. Plates were incubated for 2 h at 37 °C. The wells were washed and incubated with 200 μl of goat-anti-rabbit peroxidase immunoconjugate (Nordic) in dilution buffer (1 : 5000) for 1 h at 37 °C. For quantification of the immunoconjugate bound to the wells, an enzymatic reaction was used with o-phenylene-diamine (OPD; Sigma) as a substrate. After washing, 200 μl of substrate (0.05% OPD in citrate/phosphate buffer; 0.2 M Na_2HPO_4, 0.1 M citric acid, pH 5.0, with 0.025 % hydrogen peroxide) was added. The reaction was allowed to proceed for 20 - 30 min in the dark and was stopped by adding 50 μl of 2 M H_2SO_4. Absorbance was measured at 492 nm in a microplate reader (Titertek).

Electron microscopy

Freshly dissected CS were prefixed for 10 min in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 20 °C. The CS were then fixed for 1 h at 0 °C in this buffer containing 1% (v/v) glutaraldehyde, 0.66% (w/v) osmium tetroxide and 1.66% (w/v) potassium dichromate. Block-staining was in 2% (w/v) uranyl acetate in water. After dehydration the tissues were embedded in Spurr's resin. Ultrathin sections were contrasted with Reynold's lead citrate and examined in a Jeol 100 CX II electron microscope.

Calculations and statistics

ELISA results were evaluated using a logit transformation (Mayer-Gostan *et al*, 1991). A least squares linear regression of the transformed linear part of the standard curve was used to estimate the STC concentration of the samples. All data are presented as means $\pm$ S.E.M. The Mann-Whitney *U*-test was used for statistical evaluation. Significance was accepted for $P < 0.05$.

RESULTS

Ultrastructurally, two types of endocrine cells may be distinguished in the CS of eels: large rounded cells with large secretory granules (type-1 cells) and small cells with long cytoplasmic extensions and small secretory granules (type-2 cells; Wendelaar Bonga and Pang, 1986). STC was immunocytochemically localized in the secretory granules of both cell types indicating that both either sequester or produce stanniocalcin (Kaneko *et al.* 1989 ; Wendelaar Bonga *et al.* 1989). Because no differences were observed in the structure of the type-2 cells when eels were transferred between fresh water and sea water our present analyses focussed on the type-1 CS cells.

The ultrastructure of eel CS cells before and after transfer is shown in Fig. 1. The cells of fish acclimated to freshwater appear to be quite inactive (Fig. 1A), being characterized by an abundance of large secretory granules and relatively little granular endoplasmic reticulum (GER). Conversely the cells of fish acclimated for three weeks

to sea water contain a well developed and extensive GER and few secretory granules (Fig. 1B). Twenty-four hours after transfer of freshwater eels to sea water, the CS still did not exhibit the level of secretory activity typical of seawater acclimated fish (Fig. 1C) and the cells were similar in appearance to those of the freshwater acclimated fish containing many secretory granules. Examination of the cells of seawater fish transferred for 24 h to fresh water (Fig. 1D) or to distilled water (Fig. 1E) does not reveal noticeable differences in the extent of the GER compared to the CS-cells of the seawater acclimated fish. However, accumulation of secretory granules is observed in the cells of both groups of fish, in particular in those transferred to distilled water.

The plasma ionic and total calcium levels of freshwater and seawater eels did not differ significantly (Fig. 2). Twenty-four hours after transfer of freshwater eels to sea water, plasma total calcium was significantly increased and the change was, for the major part, due to an increase in plasma ionic calcium. Seawater eels 24 h after transfer to fresh water had plasma ionic and total calcium concentrations which were not significantly different from those of seawater acclimated eels. But transfer to distilled water produced a significant fall of plasma ionic calcium which was reflected in the decreased plasma total calcium level.

Plasma STC concentrations in freshwater and seawater eels were not significantly different. Values ranged from 0.90 to 1.30 nM (Fig. 3). Plasma STC levels of freshwater eels transferred to sea water were not significantly altered but seawater eels transferred to fresh water or distilled water showed a significant drop of plasma STC levels to values around 0.50 nM.

DISCUSSION

In this report we investigated the relation between the calcium concentration of the water and the CS activity in eels. On the short term a transfer to hypercalcic water causes hypercalcemia but does not stimulate CS activity. This observation does not favor the hypothesis of a direct regulatory role for plasma Ca^{2+} in STC secretion. Transfers to hypocalcic environments, however, induced decrease in CS activity within 24 h suggesting that a hypocalcic challenge is more adequately counteracted than a hypercalcic challenge.

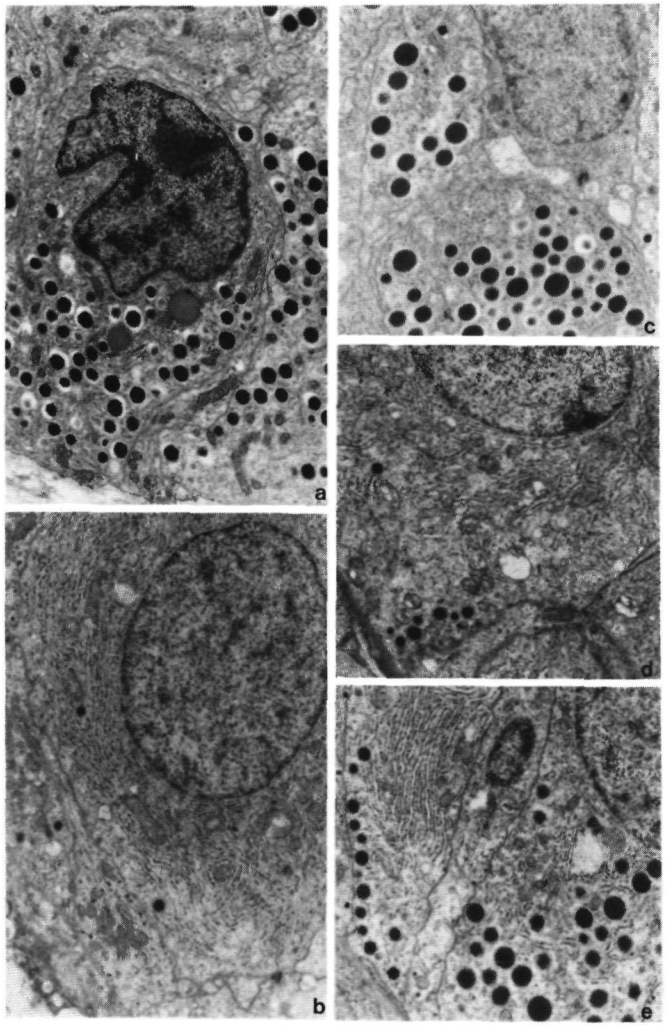

Fig. 1. *Electron micrographs of the CS of eels transferred from fresh water to fresh water (Fig. 1A) or to sea water (Fig. 1C), and the CS of eels transferred from sea water to sea water (Fig. 1B), to fresh water (Fig. 1D) or to distilled water (Fig 1E). Magnification: x 15.000.*

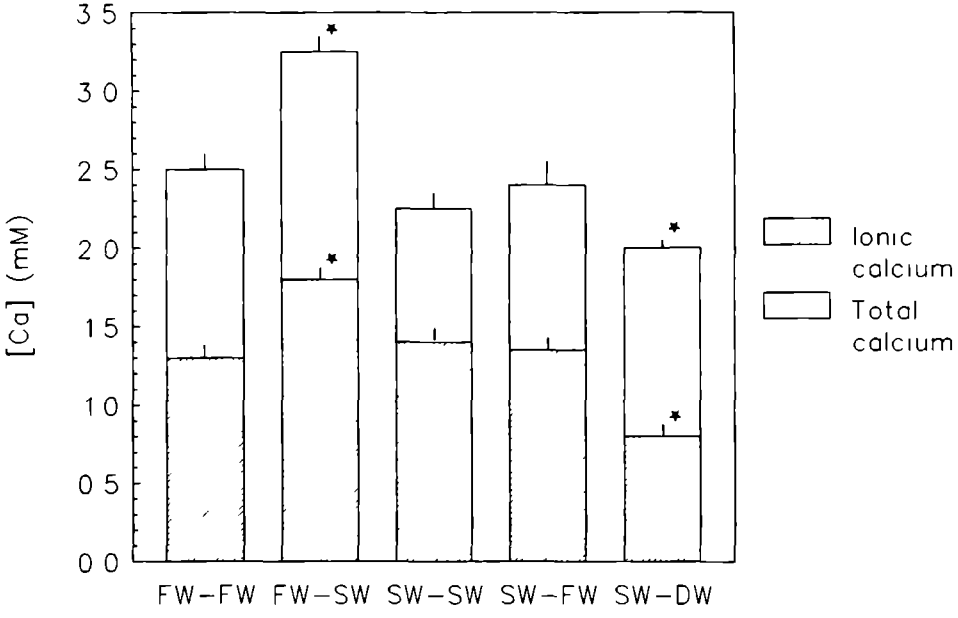

Fig. 2. *Plasma ionic and total calcium levels in eels transferred from freshwater to freshwater (FW-FW: n = 13), or to sea water (FW-SW: n = 12), and eels transferred from sea water to sea water (SW-SW: n = 9), to fresh water (SW-FW: n = 8), or to distilled water (SW-DW: n = 4). (* = significantly different from 'FW-FW' levels).*

A relation between the activity of the CS and the water calcium concentration has been suggested before. Urasa and Wendelaar Bonga (1987) reported that in tilapia a long term elevation of the water calcium concentration led to an increase in CS volume accompanied by an elevation of the plasma calcium concentration. Their results were in line with the assumption of Bailey and Fenwick (1975) that a negative feedback exists between the secretory activity of the CS and the plasma ionic calcium concentration. Further circumstantial evidence for such a relationship came from

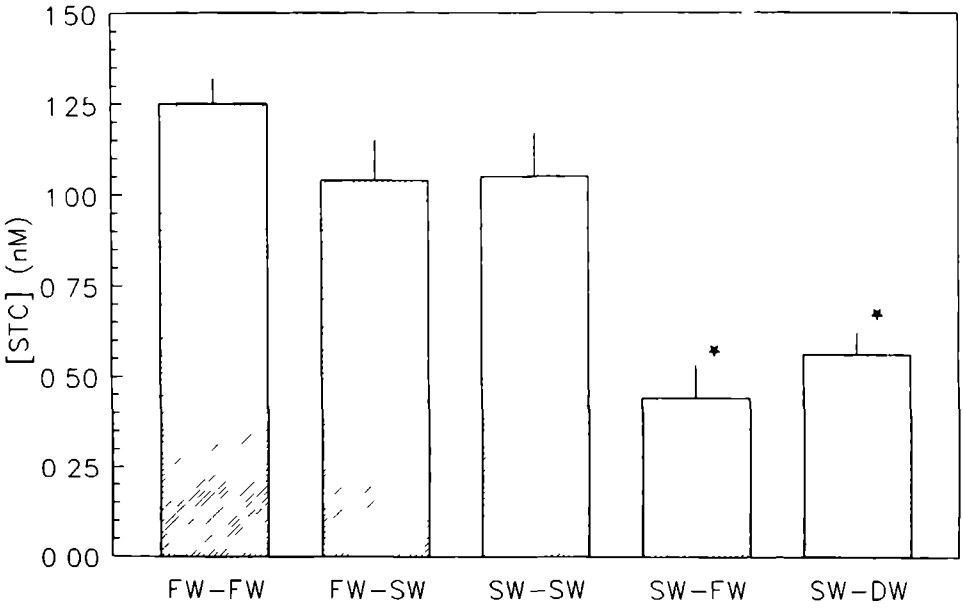

Fig. 3 : *Plasma STC levels in eels transferred from fresh water to fresh water (FW-FW: n = 13), or sea water (FW-SW: n = 5) and eels transferred from sea water to sea water (SW-SW: n = 9), fresh water (SW-FW: n = 8), or distilled water (SW-DW: n = 6). (* = significantly different from 'FW-FW' levels).*

in vitro studies by Aida *et al.* (1980) and Wagner *et al.* (1989), who concluded that increased medium calcium concentrations enhanced the depletion of CS secretory granules and the release of irSTC. However, recent *in vitro* studies by our group on eel CS showed that STC release was only altered by an increase in extracellular calcium concentration that was well above that shown to follow most hypercalcic challenges (Hanssen *et al.* 1991a).

Ultrastructural appearance

In CS cells of freshwater eels we observed a moderately developed GER and an abundance of secretory granules, indicative for a relatively inactive cell. CS cells of seawater eels were more active than those of freshwater eels as was concluded from the few secretory granules and the extensive GER. Although granule depletion has been interpreted as evidence of high secretory activity of CS cells (Cohen *et al*, 1975) this does not necessarily apply to the CS cells of all seawater-adapted euryhaline teleost fish. Granular depletion, as found in the European eel (this study) was also evident in the killifish (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980) but not in the stickleback (Wendelaar Bonga *et al.* 1976), or trout (Meats *et al.* 1978). However, in these two species a high incidence of exocytotic phenomena, another indication for high secretory activity, was observed. Ultrastructural studies on CS cells have been carried out for a number of euryhaline teleost species. In these studies it was shown that CS cells of killifish (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980), sticklebacks (Wendelaar Bonga *et al.* 1976), trout (Meats *et al.* 1978) and tilapia (Urasa and Wendelaar Bonga, 1987) become activated upon transfer of the fish from fresh water to sea water. This change in glandular activity appears to be positively correlated to the water calcium concentration (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980). Our transfer experiments show that a 24 h transfer of fresh water eel to sea water was not accompanied by an evident activation of the CS. A 24 h transfer of seawater eel to fresh water or distilled water did not effect the CS cells GER. CS cells showed an extensive GER typical for activated cells. The CS of seawater eel transferred to distilled water, and to a lesser extent the CS of seawater eel transferred to fresh water, showed accumulation of secretory granules. Accumulation of secretory granules was also found after a 24 h seawater-freshwater transfer of stickleback (Wendelaar Bonga *et al.* 1976). We conclude from our results that the activity and the degree of granulation of CS cells are related to the water calcium concentration in acclimated eels. On the short term however, transfer between high and low calcium water does not necessarily have immediate effects on CS cell activity. It may have small effects on granulation, in particular after a hypocalcic challenge. This indicates that CS cell activity in the eel is not rapidly changed after fluctuations in the water calcium concentration.

Plasma calcium levels

In fresh water eels plasma ionic and total calcium concentrations did not differ from previously reported values (1.3 mM and 2.5 mM, respectively). Plasma calcium concentrations in seawater eels did not differ significantly from those measured in freshwater eels. Also in the striped mullet no differences in plasma calcium levels were observed between fish adapted to fresh water or seawater habitats (Johnson, 1972). However, plasma calcium concentrations in trout (Meats *et al.* 1978) and tilapia (Urasa and Wendelaar Bonga, 1987) differed significantly between fish adapted to high and low calcium water. A 24 hour transfer of our freshwater eels to sea water induced a significant increase of plasma ionic and total calcium to 1.8 mM and 3.25 mM, respectively. These elevated plasma calcium levels upon transfer to sea water corroborate the report of Ogasawara and Hirano (1984) on Japanese eels, who found a transient elevation of the plasma total calcium level 24 h after transfer. We found that the plasma ionic and total calcium concentration of seawater eels after a 24 h transfer to fresh water did not change significantly. Similar observations were made by Ogasawara and Hirano (1984) who did not find significant effects on plasma total calcium in the Japanese eel after these transfers. Our seawater eels transferred for 24 h to distilled water showed a decrease in both ionic and total plasma calcium to values around 0.8 and 2.0 mM, respectively. This indicates that the eels lost the control over their branchial Ca^{2+} permeability and we predict an (increased) net Ca^{2+} efflux under these conditions. A transfer to distilled water may lead to a shift of the transepithelial potential (TEP) across the gills to (more) negative values (plasma compartment negative relative to the ambient medium). Indeed, McWilliams and Potts (1978) demonstrated a positive TEP across the gills of brown trout at ambient calcium levels of 2.0 mM and higher; the TEP shifted to negative values at ambient calcium levels of 1.0 mM and lower. As a presumed shift in the TEP to negative values would facilitate Ca^{2+} uptake from the water, the loss of calcium in our fish when challenged with low ambient Ca^{2+} levels indicates a severe disturbance of the integumental permeability to Ca^{2+} and hence a net loss of this ion. Contrary to the effects on CS cellular activity, plasma ionic and total calcium concentrations were not related to the water calcium concentration after long-term acclimation, and this suggests the presence of calcium homeostasis. Transfer of eels between high and low calcium waters as reported here provokes only transient changes in plasma calcium levels.

Plasma stanniocalcin concentrations

In freshwater eels the plasma immunoreactive STC concentration was 1.25 nM. The plasma STC concentration in seawater eels did not differ significantly from this value. These values are lower than the 2.33 nM reported recently by Mayer-Gostan *et al.* (1991) for freshwater eels. The increased plasma calcium concentration upon a 24 h transfer of freshwater eels to sea water was not accompanied by a significant increase in the plasma STC concentration and thus plasma Ca^{2+} is not the primary trigger of STC release under these conditions.

A 24 h transfer of seawater eels to fresh water or distilled water reduced the plasma STC concentration by 50%. In acclimated eels plasma STC concentrations appear to be very constant and not related to the calcium concentration of the water. A short term hypocalcic challenge, however, immediately affects the plasma STC concentration. This indicates that such a challenge inhibits the release of STC.

Conclusions

This is the first report dealing with the relationship between environmental calcium, plasma calcium, and plasma STC concentrations. We conclude that CS of freshwater eel exhibit a relatively low secretory activity and that an increase in the water calcium concentration activates the CS, but only on the long-term. A short-term increase of the water calcium concentration does not stimulate the CS despite an increase in the total and ionic plasma calcium concentration by 30% and 38%, respectively. Increased plasma calcium concentrations of this magnitude may well be within the physiological range of eel plasma. Indeed, in *in vitro* experiments we have shown that a 60% increase in the calcium concentration of the incubation medium did not affect STC release (Hanssen *et al.* 1991a). The present *in vivo* experiments confirm our conclusion based on the *in vitro* experiments that plasma calcium variations within the physiological range do not influence STC release. In the CS of seawater eels a high secretory activity was observed. This high CS activity is not reflected by an increased plasma STC level and this suggests that the metabolic clearance of STC is higher in seawater eels. When seawater eels are transferred to fresh water or distilled water, the high synthetic activity of the endocrine cells of the CS seems to be maintained for at least 24 h, but increased granulation indicates that the release of secretory material becomes

inhibited. The drop in plasma STC levels after transfer to low calcium water suggests that whereas STC clearance remains high there is a decreased STC release.

An anti-hypercalcemic role was attributed to STC by Fenwick and Forster (1972) on the basis of the ability of CS homogenates to prevent hypercalcemia after removal of the CS. Our present finding that a hypercalcemic state, evoked by transferring eels from fresh water to sea water (24 hours), is not accompanied by a stimulated release of STC indicates that a physiologically induced hypercalcemia is not a trigger for STC release. Rapid anti-hypercalcemic responses of the CS cells have so far only been reported after experimentally induced non-physiologically high plasma calcium levels. Because a direct role of calcitonin in the immediate control of plasma calcium is very unlikely in fish (Wendelaar Bonga and Pang, 1991) we conclude that regulatory mechanisms for the minute-to-minute control of plasma calcium are absent in eels, and possibly in fish in general. Apparently, plasma calcium is less tightly controlled in fish than it is in terrestrial vertebrates.

CHAPTER 6

Stanniocalcin kinetics in freshwater and seawater european eel (*Anguilla anguilla*)

SUMMARY

Stanniocalcin kinetics and hypocalcemic potency were examined in freshwater and seawater eels. The secretion rate and the metabolic clearance rate of stanniocalcin were calculated from the stanniocalcin disappearance curve after intra-arterial injection of a trout stanniocalcin-preparation. Plasma stanniocalcin concentrations in freshwater and seawater eels did not differ but the secretion rate and metabolic clearance rate in seawater eel were two times as high as in freshwater eel. Moreover, the apparent distribution space for stanniocalcin was larger in seawater than in freshwater fish. We found a much higher hypocalcemic potency of stanniocalcin in seawater eels than in freshwater eels. This may be related to an increased stanniocalcin receptor density, as indicated by an increased stanniocalcin distribution space. These observations support the notion that stanniocalcin plays a role in adaptation of fish to water with a high calcium concentration.

INTRODUCTION

Stanniocalcin (STC), a glycoprotein from the corpuscles of Stannius (CS), is the primary hormone for homeostatic control of extracellular calcium in teleost fish (Wendelaar Bonga and Pang, 1991). Removal of the CS produces a frank state of hypercalcemia in both freshwater and seawater fish (*e.g.* Fontaine, 1964; Hirano *et al.* 1981) that can be corrected by injection of purified STC (Hanssen *et al.* 1989). A long-term acclimation of fish to a high calcium environment leads to increased CS activity (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980; Flik *et al.* 1989a) but such a CS activation does not necessarily result in major changes of plasma calcium and STC concentrations (Hanssen *et al.* 1991b). Therefore, the plasma STC concentration seems to be a poor parameter for the CS activity and attention should be paid to the rate of STC metabolization.

The steady state titer of plasma STC results from the balance between the STC secretion rate (SR) and the STC metabolic clearance rate (MCR). The MCR is defined as the volume of plasma cleared from STC per unit of time (Tait, 1963). Studies on the clearance of exogenous, radiolabelled corticosteroids showed that the MCRs of cortisol in eel (Henderson *et al.* 1974; leloup-Hatey, 1974) and salmon (Donaldson and Dye, 1970; Redding *et al.* 1984) were higher in seawater- than in freshwater-acclimated fish. This fact is consistent with the increased binding of cortisol to gill tissue during seawater adaptation. Currently, our knowledge concerning the MCR and SR of peptide hormones in fish is restricted to growth hormone (GH) and prolactin. Studies on the kinetics of GH revealed no significant differences as to MCR, SR, or plasma GH concentrations between freshwater- and seawater-acclimated rainbow trout and salmon (Sakamoto *et al.* 1990; Sakamoto *et al.* 1991). However, the MCR and SR of GH of the rainbow trout were both elevated 4 days after transfer to sea water, suggesting a role for GH in the early phase of osmoregulatory adaptation to sea water (Sakamoto *et al.* 1990). The MCR, SR, and plasma concentration of prolactin were found to be elevated in freshwater acclimated salmon, which is consistent with the importance of prolactin in teleost freshwater adaptation (Sakamoto *et al.* 1991). Euryhaline fish migrating between fresh water and sea water are subject to enormous variations in the ambient calcium concentration. One may predict an increased need for STC in seawater eel because the hypercalcemic response in seawater eel following removal of the CS is much stronger than that in freshwater eel (Hirano *et al.* 1981). The ultrastructure of the CS further suggests a higher secretory

activity in seawater fish than in freshwater fish (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980; Flik *et al.* 1989a). Nevertheless we found similar STC levels and calcium concentrations in the plasma of freshwater and seawater eels (Hanssen *et al.* 1991b) and this prompted us to investigate and compare the kinetics of STC in fresh water and sea water eel.

MATERIALS AND METHODS

Animals

Freshwater European eels, weighing between 200 and 300 g, were obtained from fish dealers in the Camarque (France) and Enkhuizen (Netherlands). Fish were acclimated in 1000 L tanks containing city of Nice tap water (1.63 mM Ca^{2+}, 0.12 mM Na^{+}, 0.09 mM Cl^{-}) or artificial seawater (10 mM Ca^{2+}, 460 mM Na^{+}, 535 mM Cl^{-}) for at least three weeks before experimentation. The eels were not fed. Freshwater rainbow trout, used for the collection of the CS, were obtained from a commercial trout farm in St. Jeannet (France).

Cannulation and blood sampling

Eels were provided with a catheter (PE10, Clay Adams) via the pneumogastric artery under 0.25% MS 222 anaesthesia as described previously. Cannulated freshwater and seawater eels were allowed to recover for two days in opaque individual boxes supplied with circulating tap or seawater.

Arterial blood samples of 200 μl each were taken via the catheter 5 min before and 5, 15, 30, 45 and 60 min after injection. Additional samples were taken at 2 hour interval periods up to 10 hours and also at 24 hours hours after injection. The blood was immediately separated in plasma and cells by centrifugation (1 min at 9000 g).

Hormone preparation and administration

Freshly collected trout CS were homogenized in ice-cold saline (0.6 % NaCl) by sonication and the homogenate was centrifuged for 5 min at 9000 g. The supernatant thus obtained was injected via the catheter. The dose of STC injected was 157 μg per 100 g for freshwater fish (specific activity 150 μg per mg protein) and 126 μg per 100 g for seawater fish (specific activity 120 μg per mg protein). The carrier saline volume was 200 μl. Saline injections served as controls. To estimate the vascular distribution space of STC in the eel, the STC preparation was supplemented with [^{14}C]- mannitol (18.5 KBq per 200 μl ;specific activity 2.035 GBq per mmol; Amersham UK).

Plasma analysis

For the quantitation of plasma STC we used a competitive STC-ELISA (Mayer-Gostan *et al.* 1991) with purified trout-STC (Lafeber *et al.* 1988a) as a standard. The trout STC antiserum used (RADH-I; Kaneko *et al.* 1988), has a high degree of crossreactivity with eel STC. Plasma total calcium was measured using a commercial calcium kit (Sigma). Radioactivity was determined in 5 μl plasma samples with a Pharmacia Wallac 1410 LSA.

Calculations

An iterative, non-linear regression data analysis program for curve fitting (Leatherbarrow, 1987) was used to fit STC clearance from individual eels to a double exponential decay plus offset according to the equation:
$y = A^{(-\alpha t)} + B^{(-\beta t)} + C$, where y is the plasma STC concentration, A and B curve intercepts, α and β curve slopes, and C the offset. A similar multiexponential rate for the clearance of a peptide hormone in fish was reported for the clearance of angiotensin I in trout (Olson *et al.* 1986). The STC half-life ($T\frac{1}{2}$) is related to the curve slope as: $T\frac{1}{2}(1) = \ln 2/\alpha$ and $T\frac{1}{2}(2) = \ln 2/\beta$. The distribution space (DS) was calculated from the formula: $DS(1) = MCR/\alpha$ and $DS(2) = MCR/\beta$ (Zilversmit, 1960). To test whether the initial rapid disappearance of STC reflected distribution over the vascular bed, the disappearance of the inert volume marker ^{14}C-mannitol from the plasma was fitted to a double exponential decay: $y = A^{(-\alpha t)} + B^{(-\beta t)}$, where y is the

plasma mannitol concentration. The STC MCR, determined by integrating the area under the disappearance curve, was calculated by the method of Normand and Fortier (1970) according to MCR $= R_s.\alpha.\beta/(A.\beta + B.\alpha)$, where R_s is the injected dose of STC. The secretion rate (SR) of STC was calculated as the product of the MCR and the basal plasma hormone concentration (Tait and Burstein, 1964).

Statistics

Values for MCR, T½, SR, DS and plasma calcium of seawater and freshwater fish are presented as means $\pm$ S.E.M. Significances were assessed using the Mann-Whitney *U*-test and were accepted at $P < 0.05$.

RESULTS

The basal STC concentrations (C) before injection as well as the STC first exponential T½(1) and DS(1), second exponential T½(2) and DS(2), MCR, and SR in both freshwater and seawater eels are shown in Table 1. This table also shows the T½(1) and DS(1) of the ^{14}C-mannitol clearance in SW eels. Basal pre-injection plasma STC concentrations range between 0.71 and 1.66 nM in both freshwater and seawater fish ($P > 0.05$) and do not change after saline injections. The mean T½(1) of STC in freshwater eel is significantly higher than the mean T½(1) of STC in seawater eel while both are significantly higher than the mean T½(1) of ^{14}C-mannitol in seawater eel. Both DS(1) of STC in fresh water and sea water are significantly lower and of the same magnitude as the DS(1) of mannitol. The mean T½(2) of STC is lower in freshwater eel, whereas the MCR of STC in seawater fish is roughly two times higher. The mean STC DS(1) in freshwater and seawater eel are not significantly different but the mean STC DS(2) is significantly higher in seawater eel. The calculated STC SR is significantly higher in seawater fish than in freshwater fish

The clearance of STC from freshwater and seawater eel is shown in Fig.1. The double exponential decay in both curves indicates STC distribution over two compartments. The clearance of mannitol is shown in Fig.2. Since mannitol is a non-metabolizable sugar, the first exponential of the mannitol disappearance curve represents the distribution of mannitol in the vascular bed whereas the second

exponential most likely represents diffusion of mannitol from the fish to the water. The first exponential derived T½(1) and DS(1) of STC, therefore, are characteristic of STC distribution in the blood compartment and hence have limited value for STC metabolism.

Fig. 3 shows the effects of the STC injections on plasma total calcium in freshwater and seawater eel. A dose of 156.6 μg STC per 100 g fish causes a small but significant hypocalcemia in freshwater eels between 1 and 8 h after injection. A smaller dose of 126.1 μg STC per 100 g fish has a more pronounced effect in seawater eels where hypocalcemia is observed already 30 min after injection and is still significant 24 h after injection. Moreover, the maximum effect of STC in seawater eels is almost twice that in freshwater eels.

Table 1. *STC kinetics in eel in freshwater (FW) (n=7), and in seawater(SW) (n=6). Values are mean ± S.E.M. T½, STC half-life; C, basal STC concentration. Values of STC distribution space (DS), STC metabolic clearance rate (MCR), and STC secretion rate (SR) are per 100 g fish.*

	1st exponential A.exp(-α.t)		2nd exponential B.exp(-β.t)		C	MCR	SR
	T½(1) (min)	DS(1) (ml)	T½(2) (min)	DS(2) (ml)	(nM)	(ml/min)	(ng/min)
STC FW eel	12.5[a,b] ±0.6	2.0[b] ±0.2	121.8[a] ±5.1	19.4[a] ±1.0	0.89 ±0.05	0.111[a] ±0.006	5.6[a]±0.5
STC SW eel	8.4[b] ±0.4	2.2[b] ±0.1	103.6 ±10.8	28.0 ±2.2	0.93 ±0.06	0.189 ±0.015	9.8±1.3
Mannitol SW eel	7.7 ±0.9	4.3 ±0.2					

a = significantly different from STC SW value

b = significantly different from mannitol SW value

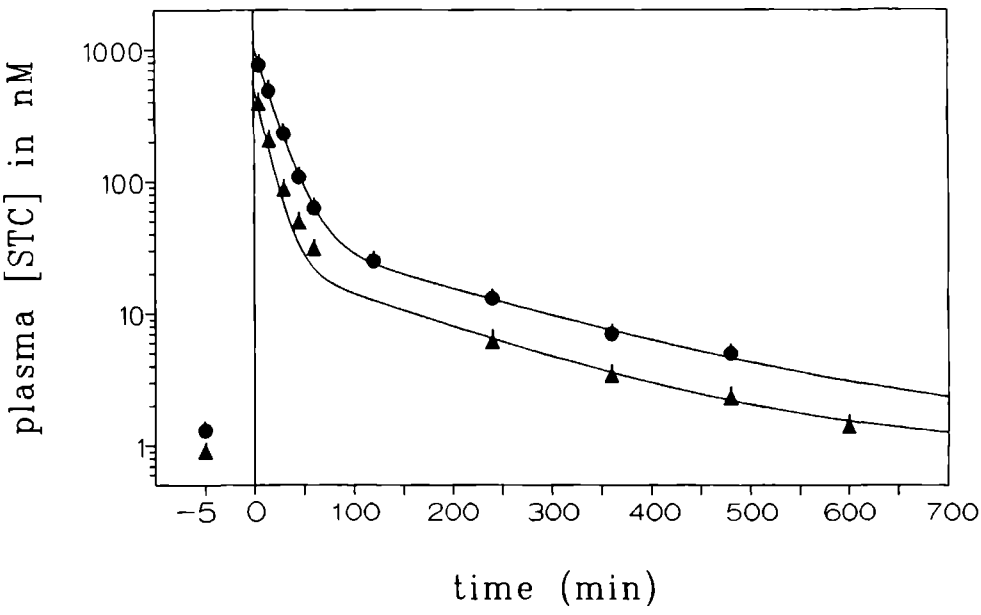

FIG. 1. *Plasma clearance curves of trout STC after injection into freshwater eel (●) (n=7), and seawater eel (▲) (n=6). Values are means ± S.E.M.*

DISCUSSION

This study presents the kinetics of the calcium-regulating hormone STC in fish adapted to fresh water or sea water. We found a significantly higher STC MCR and SR in seawater eels than in freshwater eels, whereas plasma STC concentrations did not differ significantly.

There is remarkable similarity between the initial distribution of mannitol and STC, the volume in which the distribution occurs, and the actual blood volume of the eel (3.5 ml / 100 g; Takei, 1988). Therefore, we conclude that the values for DS(1) of mannitol and STC reflect the blood compartment space over which the hormone initially is distributed. The early rapid decrease in plasma STC is due to dilution.

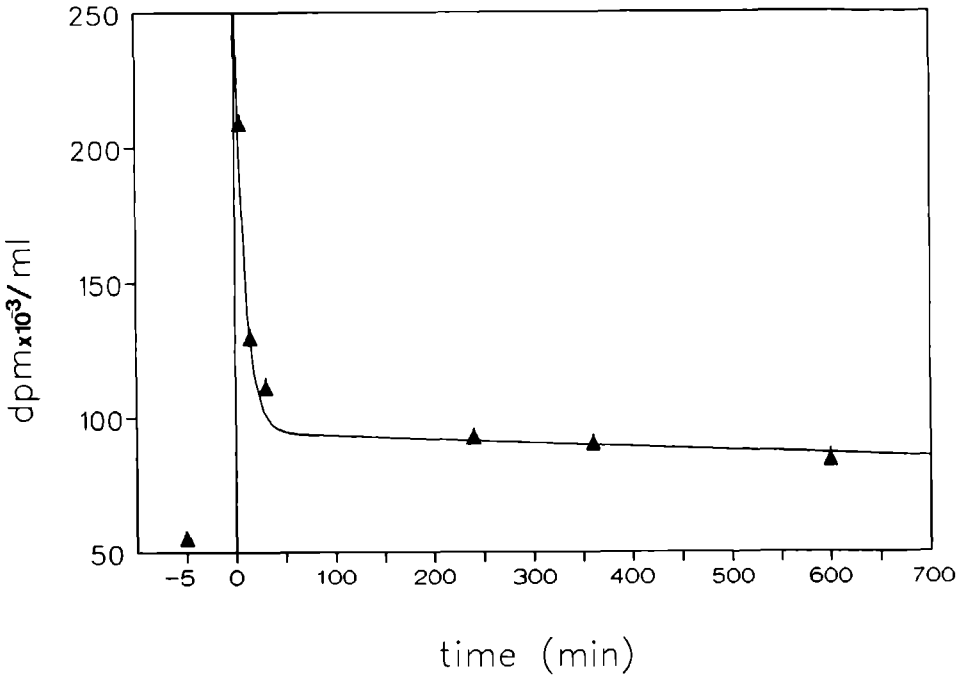

FIG. 2. *Plasma clearance curve of ^{14}C-mannitol after injection into seawater eel (n=6). Values are mean ± S.E.M.*

Similar results were reported for the clearance of angiotensin I in trout (Olson *et al.* 1986). Studies on hormone kinetics have largely been limited to the estimation of the plasma T½ from the disappearance curve of the administered hormone. However, since the T½ does not reflect the space available for STC distribution (Owens *et al.* 1973) T½ is a poor estimate of STC metabolism. The MCR, which is determined by both T½ and DS of STC, is a more appropriate parameter for STC kinetics. The MCR of STC we calculated in seawater eels (0.189 ml/min/100 g) is almost twice that of STC in freshwater eels (0.111 ml/ min/100 g), which indicates that plasma STC clearance is much faster in seawater eels. A similar higher MCR in seawater than in

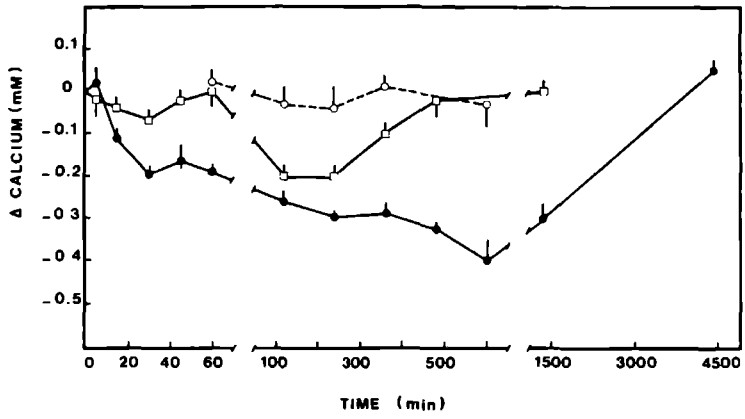

FIG. 3. *Plasma total calcium levels after injection of trout STC into freshwater eel (○) (n=7), and seawater eel (●) (n=6). Saline injection in seawater eel (○) (n=4) served as control. Values are means ± S.E.M. and are shown as differences compared with preinjection levels.*

freshwater adapted fish was reported for corticosteroids in eel (0.033 ml/min/100 g in freshwater *vs* 0.047 ml/min/100 g in sea water; Henderson *et al.* 1974) and in salmon (0.097 ml/min/100g in fresh water to 0.140 ml/min/100 g in sea water; Redding *et al.* 1984). The calculated STC DS(2) in seawater eels exceeds the DS(2) in freshwater eels. This increased DS(2) could represent increased degradation in the clearance organs or an increased binding to STC receptors. For peptide hormones, kinetic studies revealed no significant difference in MCR for growth hormone between freshwater and seawater adapted trout (Sakamoto *et al.* 1990) and salmon (Sakamoto *et al.* 1991), whereas the MCR of prolactin in salmon increased after transfer of the fish from sea water (0.333 ml/min/100 g) to fresh water (0.433 ml/min/100 g; Sakamoto *et al.* 1991). Therefore, it is unlikely that an overall non-specific activation of organs which help clearing peptide hormones from the blood (liver, gut, kidney), explains the increased MCR of STC in seawater fish. The increased prolactin metabolism reported in freshwater salmon (Sakamoto *et al.* 1991) is in accordance with the role of prolactin in the hydromineral control of freshwater fish (Wendelaar Bonga

and Pang, 1989). The principal target of STC probably is the branchial chloride cell. STC exerts its function by controlling transcellular calcium transport (Wendelaar Bonga and Pang, 1991). The increased STC DS(2) we found in seawater eel might result from the increase in number and volume of chloride cells that occurs after transfer of freshwater adapted eel to sea water (Pisam *et al.* 1987). Our observation that plasma calcium levels are similar in freshwater and seawater eel, irrespective of the almost tenfold difference in water calcium concentration between fresh water and sea water, indicates that the activity of the endocrine glands involved in the hypocalcemic control of the eel must be much higher in sea water than in fresh water. The CS are the only endocrine glands producing a potent hypocalcemic hormone in fish, *i.e.* STC. The present observations indeed indicate increased activity of the CS in seawater eel. The secretion rate of STC is the product of MCR of STC and the basal STC concentration. Because the MCR of STC is higher in seawater than in freshwater eel and the plasma STC concentration is similar, the SR of STC is higher in seawater eel. This is in line with ultrastructural and biochemical observations on the CS, showing a high synthetic and secretory activity in seawater fish, when compared with freshwater fish (Wendelaar Bonga *et al.* 1980; Hanssen *et al.* 1991b).

In summary, the present results demonstrate that the clearance of STC from plasma is faster in seawater than in freshwater eel. This explains why the plasma STC levels are similar in freshwater and seawater eel even though the synthetic and release activities of the CS are known to be higher in seawater eel.

CHAPTER 7

Cholinergic modulation of stanniocalcin secretion

SUMMARY

The acetylcholine agonist carbachol stimulates stanniocalcin release *in vitro* from the corpuscles of Stannius of the freshwater-adapted eel (*Anguilla anguilla*), the muscarinic acetylcholine-receptor antagonist atropine blocks this stimulation. This indicates that a cholinergic mechanism is involved in freshwater control of stanniocalcin release. In seawater-adapted eels carbachol does not stimulate stanniocalcin release, which strongly suggests that a cholinergically recruitable stanniocalcin pool in the corpuscles of Stannius is lacking.

INTRODUCTION

Stanniocalcin (STC), the principal secretory product of the corpuscles of Stannius (CS) of teleost fish is a fast-acting, calcium influx inhibiting hormone (Wagner *et al.* 1986; Lafeber and Perry, 1988) specifically regulating the plasma ionized calcium fraction (Hanssen *et al.* 1989). The CS secretory activity of fish is related to the calcium concentration of the ambient water (Cohen *et al.* 1975; Wendelaar Bonga *et al.* 1980) but this relation is not reflected by immediate alterations in plasma STC and calcium levels following changes in water calcium (Hanssen *et al.* 1991b). Variations in the extracellular plasma Ca^{2+} concentration were shown to induce STC release *in vivo* (Flik *et al.* 1989a) and *in vitro* (Wagner *et al.* 1989; Hanssen *et al.* 1991a), suggesting a feedback relationship between plasma ionic calcium and STC release. However, we found that in CS of the eel (*Anguilla anguilla)* a STC release *in vitro* was stimulated only by Ca^{2+} concentrations above 2.5 mM, which is well above the physiological plasma Ca^{2+} range (Hanssen *et al.* 1991a). This finding raises doubts as to the significance of the plasma calcium concentration for the regulation of STC release.

A nervous modulation of CS secretory activity has been suggested as CS are richly innervated (Krishnamurthy and Bern, 1971; Belsare, 1973; Unsicker *et al.* 1977; Wendelaar Bonga *et al.* 1977). In a number of species, vasculo-ganglionic units consisting of ganglion cells, nerve fibers and blood vessels, were found either in close proximity or inside the parenchyma of the CS (Heyl, 1970; Belsare, 1973). Nerve fibers associated with ganglion-like groups of neurons penetrate the CS capsule along with blood vessels but do not make synaptic contact with CS secretory cells (Krishnamurthy and Bern; 1971, Wendelaar Bonga *et al.* 1977; Bhattacharyya and Butler, 1978). The CS penetrating nerves proved to be sympathetic in nature (Young, 1931; Wendelaar Bonga *et al.* 1977) and therefore both adrenergic and cholinergic neurotransmitters might influence STC secretion. Preliminary results (G. Flik, personal communication) have shown increased exocytotic activity and STC release in the CS cells of rainbow trout exposed to media containing the acetylcholine agonist carbachol, whereas media containing adrenergic agonists did not induce secretory activity. Therefore, we have aimed to substantiate the hypothesis that STC secretion by the CS of the eel is under cholinergic control.

MATERIAL AND METHODS

Animals

European eels, weighing between 200 and 300 g, were kept in 1000-liter tanks with either circulating fresh water (Ca^{2+}, 0.7 mM; Na^+, 1.9 mM; Cl^-, 3.1 mM; Mg^{2+}, 0.2 mM), or sea water (Ca^{2+}, 12.5 mM; Na^+, 510 mM; Cl^-, 555 mM; Mg^{2+}, 58 mM),at 15 °C. The eels were acclimated to fresh water or sea water for at least 14 days before experiments started. No food was supplied. Freshly dissected eel CS were freed from connective tissue and collected in Hanks' balanced salt solution (HBSS; Flow laboratories).

Incubations

Incubations were performed as described before (Hanssen *et al.* 1991a), with some modifications. Control incubation media were prepared by adding $CaCl_2$ (final Ca^{2+} concentration, 1.25 mM) and ascorbic acid (1 mg/l) to Ca^{2+}-free HBSS (pH 7.4). To experimental incubation media, carbachol (final concentration, 10^{-5} M) and/or atropine (final concentration, 10^{-6} M) were added. Two CS from different eels were pre-incubated for 3 hr at 28 °C in an incubation vessel containing 50 μl control incubation medium supplemented with 925 KBq [^{3}H]-leucine (Amersham; specific activity 5.2 TBq/mmol). CS were then washed in 2 ml incubation medium (3 times) and incubated for 30 min in either control incubation medium (control and carbachol incubations) or medium supplemented with 10^{-6} M atropine (atropine/carbachol and atropine incubations). STC release was studied in a final 2.5 hr incubation period, in 200 μl incubation medium. Incubation media were collected and CS were homogenized in 250 μl 0.1 M acetic acid. Part of the incubation media and CS homogenates (100 μl) was set aside for enzyme linked immunosorbent assay (ELISA). The remainder of the media was precipitated with 10% TCA (4°C, 16 hrs), the remainder of the CS homogenates was lyophilized. Radioactivity in media and homogenates was determined with a LKB Rackbeta LSA. A 30 kD product, as judged from SDS-PAGE, could be identified as eel STC (Flik *et al.* 1989) and was the dominant product secreted by the CS.

ELISA

A competitive ELISA, developed by Mayer-Gostan *et al.* (1991), was used for STC quantitation. In short, microtiter plates were coated with 1.85 nM trout STC and stored at 4 °C. For antigen/antibody competition equal volumes of antigen and diluted antiserum (RADH-1; *cf* Kaneko *et al.* 1988; dilution 1:40,000) were incubated at 20 °C for 16 hours. Trout STC was used as a standard. The wells of the coated plates were blocked with porc serum and incubated with the antigen/antiserum mixture. Subsequently, the wells were incubated with goat-anti-rabbit peroxidase immunoconjugate (Nordic; 1 : 5000). An enzymatic reaction with o-phenylene-diamine (OPD; Sigma) as a substrate was used for quantification. The reaction was stopped with 4N H_2SO_4 and absorbance was measured at 492 nm in a microplate reader (Biorad).

Calculations and Statistics

The data of *in vitro* incubations are presented as percentages STC released into the incubation medium. Data are presented as means $\pm$ SEM. The Mann-Whitney U-test was used for statistical evaluation. Significance was accepted at $P < 0.05$.

RESULTS

Fig. 1 shows that 10^{-1} M carbachol stimulates the *in vitro* release of both newly synthesized and total immunoreactive STC from the CS of freshwater-adapted eels. Addition of 10^{-6} M atropine blocks completely this stimulation. Atropine alone has no significant effect. A comparison between the secretion of newly synthesized and total STC from freshwater-adapted or seawater-adapted eel CS is shown in Fig. 2. In control incubations the percentual release of newly synthesized and total STC from the CS of freshwater-adapted and sewater-adapted eels is not significantly different despite marked differences in the absolute contents of newly synthesized STC (about 143,000 dpm in freshwater-adapted eel *vs* about 1000,000 dpm in seawater-adapted eel) and total STC (280 ng/μg protein in freshwater-adapted eel *vs* 61 ng/μg protein in seawater-adapted eel). In freshwater-adapted eel CS, 10^{-5} M carbachol stimulates the

release of newly synthesized STC 2-fold and of total STC 1.7-fold. No such stimulation was observed in seawater eel CS treated in an identical manner.

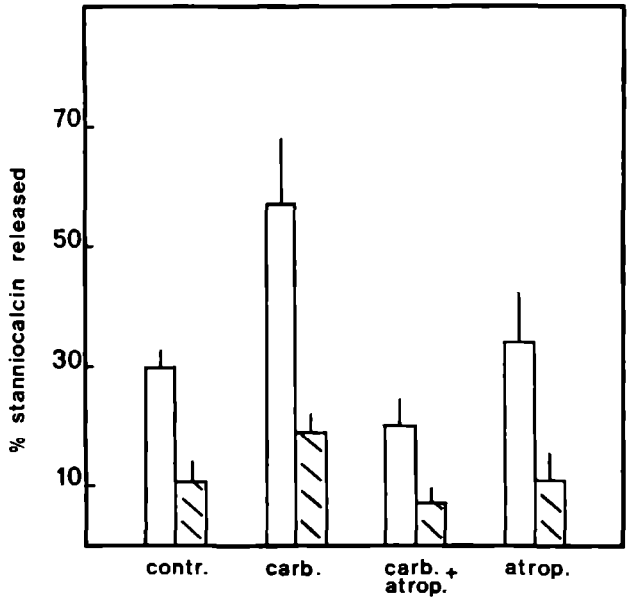

Fig. 1. *Effect of carbachol (10^{-5} M) and/or atropine (10^{-6} M) on the in vitro release of newly synthesized (□) and total immunoreactive (▨) STC from freshwater eel CS. STC in incubation medium and in CS homogenate together is designated 100%. (N = 4).*

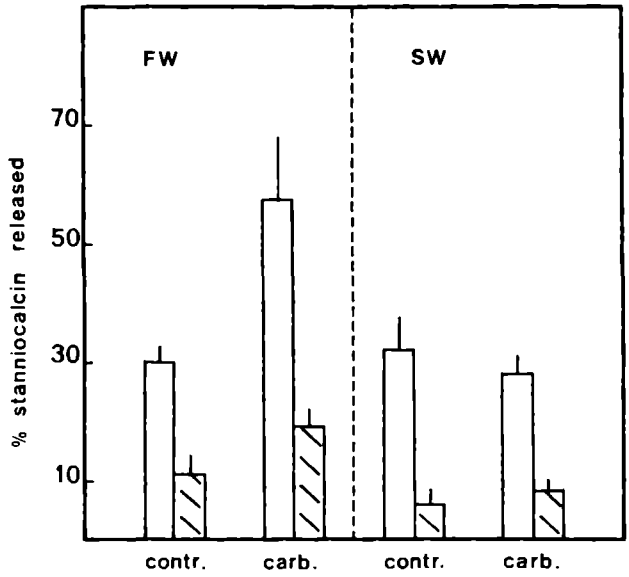

Fig. 2. *Effect of carbachol (10^{-5} M) on the release in vitro of newly synthesized (□) and total immunoreactive (▨) STC from freshwater (FW) and seawater (SW) eel CS. STC in incubation medium and in CS homogenate together is designated 100%. (N = 4).*

DISCUSSION

In FW eel CS carbachol stimulates the release of newly synthesized as well as total STC *in vitro*. This result implies a function of acetylcholine as stimulator of the release of STC in these fish. Although a nervous innervation of the CS in a number of teleost species has been demonstrated (Krishnamurthy and Bern, 1971; Belsare, 1973; Unsicker *et al.* 1977; Wendelaar Bonga *et al.* 1977), the nature of this neural control is poorly understood. In eel and killifish, autotransplantation of CS does not affect the ability of the CS to regulate plasma calcium 10 to 14 days after transplantation (Fenwick and Forster, 1972; Schreibman and Pang, 1975). Therefore, the significance of ganglia in the control of CS secretory activity is not evident. However, CS transplants are well vascularized and may therefore be (re)innervated, an aspect not addressed to in any study so far. Unsicker *et al.* (1977) found in rainbow trout a

pronounced adrenergic innervation of the CS and adjacent parts of the kidney. Blood vessels penetrating the CS were accompanied by bundles of axons and noradrenaline-containing cells. The presence of considerable amounts of noradrenaline, adrenaline and 5-hydroxytryptamine was demonstrated. Adrenaline, noradrenaline and dopamine were also detected in eel CS (Hathaway and Epple, 1989) but the amount of these neurotransmitters does not exceed that in teleost peripheral organs in general. Considering the absence of direct innervation of CS cells in the eel we assume that adrenergic activities in the CS are associated with the vascular bed of the gland.

Up to now no cholinergic innervation or presence of acetylcholine has been demonstrated in the CS. Our results in frehwater-adapted eel CS show a cholinergic stimulation of STC release that can be blocked by the muscarinic acetylcholine antagonist atropine. This indicates the presence of a muscarinic acetylcholine receptor on CS cells. The mechanism by which carbachol stimulates STC secretion is not known. Activation of an acetylcholine-receptor operated calcium channel in the cellular plasma membrane of a clonal nerve cell line was shown to cause a rise in the cytosolic calcium concentration which is sufficient to evoke a secretory response (Stallcup, 1979). However, the acetylcholine receptor involved in the control of calcium influx proved to be of the nicotinic and not of the muscarinic type (Meldolesi and Pozzan, 1987). Secretion of newly synthesized and total STC from freshwater eel CS is also stimulated by very high extracellular calcium concentrations (2.5-3.75 mM) and by the calcium ionophore calimycin, which indicates that a calcium influx is required to induce secretion (Hanssen *et al.* 1991a). However, the preferential release of stored STC in these experiments is not consistent with our present results on carbachol-stimulated release. Whereas in freshwater-adapted eels the cholinergic stimulation of the release of newly synthesized STC is similar to the calcium influx-induced release, the percentage of total STC released is only half that of the percentage total STC released upon a calcium influx. It is therefore unlikely that a acetylcholine-mediated calcium entry into the cell represents the mechanism that stimulates the release of stored STC. This conclusion supports the hypothesis that there are more, individually recruitable, STC pools (Hanssen *et al.* 1991a) and indicates that upon carbachol challenge not all STC pools are involved in STC secretion. Electron micrographs of the CS cells of seawater-adapted eels show only few secretory granules and an extensive rough endoplasmatic reticulum, indicative for an active synthesizing cell (Hanssen *et al.* 1991b). Despite the presence in seawater-adapted eel CS of approximately eight times the amount of newly synthesized STC of freshwater-adapted eel CS, the newly

synthesized STC in seawater eel CS does not appear to be recruitable by cholinergic stimulation. Therfeore, the absence of a cholinergic stimulation in the CS of seawater-adapted eel strongly suggests the absence of a cholinergic receptor in such animals.

CHAPTER 8

General discussion and summary

The work presented in this thesis was carried out in order to study the control of stanniocalcin synthesis and secretion and to establish its role in the maintenance of calcium homeostasis in teleost fish. Stanniocalcin is the dominant calcium regulating hormone in fish and therefore, the major part of this thesis deals with the mutual relation between plasma calcium and stanniocalcin concentrations and its relevance for the functional aspects of anti-hypercalcemic control.

regulation of plasma ionic calcium

In chapter 2 we show that stanniocalcin exerts its function as a calcium regulating hormone by controlling plasma ionic calcium, a physiologically important calcium compartment in the body. Removal of the corpuscles of Stannius (CS) results in a rise of both plasma ionic and protein-bound calcium, which implies an increased binding of ionic calcium to plasma calcium binding proteins. Injection of stanniocalcin in stanniectomized eels induces a rapid decrease in plasma total calcium that is almost totally accounted for by a decrease in plasma ionic calcium. This indicates that stanniocalcin inhibits the uptake of ionic calcium. The sustained elevated plasma calcium levels 40 days after stanniectomy are indicative for the absence of potent hormones with anti-hypercalcemic effects other than stanniocalcin. This implies that an anti-hypercalcemic function for calcitonin in fish is unlikely, in contrast to suggestions in the literature.

stanniocalcin synthesis

Trout stanniocalcin is synthesized *in vitro* as a 56-kDa glycoprotein processed from a 64-kDa precursor (chapter 3). The protein is probably homodimeric in nature and contains a 5- to 6-kDa glycomoiety which has no antigenic sites for the trout RADH-1 antiserum. The identification of several isoforms of stanniocalcin may indicate the presence of several stages of maturation. A $CaCl_2$ injection induces an increased stanniocalcin synthesis but whether this is indicative for a regulatory role of plasma Ca^{2+} in stanniocalcin synthesis *in vivo* remains to be elucidated.

stanniocalcin secretion; regulation by plasma Ca^{2+}

The anti-hypercalcemic action of STC opens the possibility that the plasma Ca^{2+} concentration is an important factor for the control of STC secretion. We therefore investigated the possible regulatory function of plasma Ca^{2+}in fish with regard to the release of STC. The results show that secretion of stanniocalcin *in vitro* (chapter 3) and *in vivo* (from the CS into the blood; chapter 4), is stimulated following $CaCl_2$ injection. This clearly shows that an elevation of the plasma ionic calcium concentration induces an enhanced stanniocalcin secretion. However, this stimulatory effect does not allow the conclusion that stanniocalcin secretion *in vivo* is directly controlled by plasma ionic calcium because the plasma ionic calcium concentration induced by a $CaCl_2$ injection (2.5 - 3.75 mM) is much higher than may be expected to occur under normal conditions. In addition, whereas a high plasma ionic calcium concentration as measured after $CaCl_2$ injection induces stanniocalcin release *in vitro*, concentrations corresponding with physiologically relevant ionic calcium levels or lower (0 - 1.5 mM) have no effect (chapter 4). Therefore, the physiological relevance of the $CaCl_2$-injection experiments remains to be demonstrated. To test whether physiologically relevant plasma calcium variations effect stanniocalcin secretion *in vivo* we transferred eels between fresh water and sea water, the most extreme physiological Ca^{2+} challenge that the fish may be expected to encounter during their lifetime. A 24 hour transfer of freshwater acclimated eels to seawater increases plasma ionic calcium from 1.5 to 1.8 mM. However, this is not accompanied by an increased plasma stanniocalcin concentration nor by an activation of stanniocalcin synthesis and release as judged from the ultrastructural appearance of the CS cells (chapter 5). This suggests that the results of the $CaCl_2$-induced surge of plasma ionic calcium have no physiological relevance for the regulation of stanniocalcin secretion *in vivo*. We conclude that changes in CS activity do not appear rapidly after a physiological hypercalcemic challenge and that a direct control of stanniocalcin secretion by plasma ionic calcium *in vivo* is absent. This conclusion is in contrast with the conclusions of other authors, who all used non-physiologically high Ca^{2+} concentrations in their experiments.

nervous modulation of stanniocalcin secretion

The absence of clear effects of changes in plasma Ca^{2+} on STC secretion raises the question whether the STC secretion is under nervous control. In chapter 7 we demonstrate the effect of carbachol, a cholinergic agonist, on stanniocalcin secretion. In freshwater eel CS carbachol stimulates stanniocalcin secretion *in vitro*, a proces that can be blocked by the muscarinic cholinergic antagonist atropine. This implies a function for acetylcholine in the release of stanniocalcin in these fish and indicates the presence of a muscarinic acetylcholine receptor on stanniocalcin secreting CS cells. Carbachol does not stimulate stanniocalcin release in seawater eel which might indicate the absence of an acetylcholine receptor on CS cells of seawater eel.

stanniocalcin secretory pools

In vitro incubations of freshwater eel CS in high-Ca^{2+} media (2.5 - 3.75 mM) induce the release of both newly synthesized and total stanniocalcin. However, stored stanniocalcin is released preferentially. Calimycin A23187 mimics the stimulatory effect of high-Ca^{2+} and also induces a preferential release of stored stanniocalcin (chapter 4). This indicates that there are at least two stanniocalcin secretory pools in CS cells which are individually recruitable. Stored stanniocalcin is released following an increased Ca^{2+}-influx in the CS cells whereas the release of newly synthesized stanniocalcin appears to be less dependent of an external stimulus. The carbachol stimulated stanniocalcin release from freshwater eel CS cells is not characterized by a preferential release of stored stanniocalcin (chapter 7). It is therefore unlikely that activation of an acetylcholine receptor on the CS cells induces a Ca^{2+}-influx into the cell.

stanniocalcin secretion and kinetics; relation with the water calcium level

Ultrastructurally, CS cells appear to be more active in eels acclimated to sea water than in eels acclimated to fresh water. This suggests a relation between CS cell activity and the water calcium concentration in acclimated eels. However, plasma calcium and stanniocalcin concentrations in these fish do not differ (chapter 5). This implies that the plasma stanniocalcin concentration is not the only indicator for the CS secretory

activity in these fish, and that the stanniocalcin turnover should be considered. Indeed, examination of the stanniocalcin kinetics in freshwater- and seawater-acclimated eel shows a metabolic clearance rate of stanniocalcin in seawater-acclimated fish that nearly doubles that in freshwater-acclimated fish (chapter 6). Moreover, the apparent distribution space of stanniocalcin is much larger, and the hypocalcemic potency of stanniocalcin much higher in seawater-acclimated eels. This might result from the well known increased number and volume of the chloride cells, the principal stanniocalcin target, or from an increase or modulation of stanniocalcin receptors on these cells in seawater-acclimated fish.

Whereas a 24 hour transfer of freshwater-acclimated eels to sea water has no immediate effect on CS secretory activity, a 24 hour transfer of seawater-acclimated eels to fresh water reduces the secretory activity and induces a drop in plasma calcium and plasma STC concentrations (chapter 5). The drop in plasma stanniocalcin levels in the latter fish can be explained by the assumption that these animals still have the high stanniocalcin clearance rate that characterizes seawater-acclimated fish, combined with the decreased stanniocalcin release typical for fish in fresh water.

Fish acclimated to fresh water may face water ionic calcium levels as low as one tenth that of the blood while fish acclimated to sea water face water ionic calcium levels ten times higher than that of the blood. Therefore, a prominent role for a hypercalcemic hormone is more likely in freshwater-acclimated fish, whereas seawater-acclimated fish need a dominant hypocalcemic hormone. Indeed, the most probable hypercalcemic hormone in fish, prolactin, is more prominent in freshwater-acclimated fish and is therefore considered a freshwater hormone.

Our results indicate that in fish acclimated to sea water a transfer to fresh water or distilled water is rapidly counteracted by changes in stanniocalcin secretion, whereas a transfer to sea water of freshwater-acclimated fish has no immediate effect. Furthermore, the high synthetic and secretory activity of the CS cells and the high metabolic clearance rate of stanniocalcin in seawater-acclimated fish, compared to freshwater-acclimated fish, indicate that maintenance of the calcium homeostasis by stanniocalcin in sea water requires more metabolic energy than in fresh water. The increased stanniocalcin distribution space and the much greater hypocalcemic potency of stanniocalcin in seawater-acclimated fish suggests a more pronounced role of stanniocalcin in the maintenance of calcium homeostasis. We postulate that, whereas prolactin is considered to be a fresh water calcitropic hormone, stanniocalcin predominantly is a sea water calcitropic hormone.

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SAMENVATTING

Gewervelde dieren kunnen zonder calcium niet adequaat functioneren en zijn daarom gedurende hun hele leven aangewezen op de opname van calcium van buiten het lichaam. De opname van calcium, die bij landdieren via het voedsel plaats vindt, gebeurt bij vissen voornamelijk via opname uit het water door de kieuwen. Controle van de calciumopname via de kieuwen is daarom voor de vis een essentieel proces. De hormonale controle van de calciumopname via de kieuwen, en daarmee samenhangend de controle van de calciumhomeostase, wordt bij beenvissen gedomineerd door het hormoon stanniocalcine, dat geproduceerd wordt in de lichaampjes van Stannius. Met de in dit proefschrift beschreven experimenten is getracht een beeld te verkrijgen van de synthese en de secretie van stanniocalcine en van de wijze waarop deze gereguleerd worden. De aandacht is hierbij speciaal uitgegaan naar de relatie tussen de stanniocalcine-secretie en de concentratie van het ionogene calcium in het bloedplasma. Verder is getracht inzicht te verkrijgen in de relatie tussen de calciumconcentraties in water en plasma, de stanniocalcine-secretie, en daarmee samenhangend de calcium-regulerende potentie van het hormoon in zoet water en zeewater.

stanniocalcine-synthese

Het *in vitro* nieuw-gesynthetiseerde stanniocalcine in de forel is een glycoprotëine van 56 kDa dat afkomstig is van een precursor molecule van 64 kDa (Hoofdstuk 3). De 5 tot 6 kDa grote glycogroep wordt, in tegenstelling tot het gehele molecule, niet herkend door een specifiek forel-stanniocalcine antilichaam. Het hormoon, dat waarschijnlijk in de natieve vorm een homo-dimeer is, komt voor in verschillende iso-vormen. Een intra-peritoneale injectie van $CaCl_2$ in de vis veroorzaakt een stimulatie van de stanniocalcine-synthese. In hoeverre deze toename relevant is voor de *in vivo* relatie tussen de Ca^{2+} concentratie van het plasma en de stanniocalcine-synthese blijft vooralsnog onduidelijk en wordt verder onderzocht.

Het operatief verwijderen van de lichaampjes van Stannius (stanniëctomie) leidt tot een aanhoudende hypercalcemie die gekenmerkt wordt door een toename van zowel het ionogene calcium als het eiwit-gebonden calcium in het plasma (Hoofdstuk 2). Het langdurige karakter van deze hypercalcemie wijst erop dat er naast stanniocalcine geen andere potente anti-hypercalcemische hormonen aanwezig zijn in de vis. Injectie van stanniocalcine in gestanniëctomeerde vissen heeft een verlaging van de calcium-spiegels van het plasma tot gevolg die volledig toe te schrijven is aan een verlaging van de concentratie van het ionogene calcium in het plasma. Dit wijst erop dat de functionele rol van stanniocalcine voornamelijk berust op de regulatie van het ionogene calcium in het plasma, gemediëerd door een remming van de influx van ionogeen calcium.

In vitro incubatie van de lichaampjes van Stannius van $CaCl_2$ geïnjecteerde vissen veroorzaakt niet alleen een stimulatie van de stanniocalcine-synthese maar ook een stimulatie van de stanniocalcine-secretie (Hoofdstuk 3). Ook *in vivo* wordt de secretie van stanniocalcine (van de lichaampjes van Stannius naar het bloed) gestimuleerd na $CaCl_2$-injectie (Hoofdstuk 4). Dit wijst erop dat de concentratie van het ionogene calcium in het plasma de stanniocalcine-secretie kan beïnvloeden. De fysiologische relevantie van deze $CaCl_2$ experimenten lijkt echter beperkt omdat de concentratie van het ionogene calcium in het plasma na $CaCl_2$-injectie (2,5 - 3,75 mM) veel hoger is dan de concentratie van het ionogene calcium in het plasma van niet geïnjecteerde dieren (1,25 - 1,50 mM). *In vitro* incubaties van Stannius lichaampjes in media met deze hoge calcium concentraties induceren inderdaad stanniocalcine-secretie terwijl incubaties in media met normale of nog lagere calcium- concentraties geen stimulatie van de stanniocalcine-secretie laten zien (Hoofdstuk 4). Teneinde meer inzicht te verkrijgen in de effecten van de concentratie van het ionogene calcium in het plasma op de stanniocalcine-secretie onder natuurlijke condities werden palingen overgebracht van zoet water naar zeewater. Een dergelijke kort durende transfer veroorzaakt een stijging van de concentratie van het ionogene calcium in het plasma tot 1.8 mM (Hoofdstuk 5). Deze stijging, die de grootste is die de paling in de natuur kan overkomen, heeft *in vitro* geen meetbaar effect op de secretie van stanniocalcine. De stimulatie van de stanniocalcine-secretie na $CaCl_2$-injectie is daarom fysiologisch niet-relevant, omdat de hiervoor benodigde stijging van de concentratie van het ionogene calcium van het bloedplasma onder natuurlijke omstandigheden zeer waarschijnlijk niet voor zal komen. Een directe regulatie van de stanniocalcine-secretie door het ionogene calcium in het plasma lijkt dan ook uitgesloten.

nerveuze modulatie en intra-cellulaire stanniocalcine pools

Tijdens *in vitro* incubaties van de lichaampjes van Stannius van zoetwater-palingen met de acetylcholine-agonist carbachol wordt de secretie van stanniocalcine gestimuleerd, een proces dat geblokkeerd wordt na toevoeging van atropine, een muscarine-acetylcholine receptor-antagonist (Hoofdstuk 7). Dit wijst op een cholinerge nerveuze controle van de stanniocalcine-secretie en de mogelijke aanwezigheid van een muscarine-acetylcholine receptor op de stanniocalcine-secreterende cellen. Een dergelijk stimulerend effect van carbachol is niet gevonden na incubatie van de lichaampjes van Stannius van zeewater-palingen en daarom is een cholinerge controle van de stanniocalcine-secretie in zeewater niet waarschijnlijk.

Incubatie van de lichaampjes van Stannius in media met een hoge niet-fysiologische calcium concentratie (2,5 - 3,75 mM) of in media waaraan de calcium ionofoor calimycine is toegevoegd resulteert in een stimulatie van de secretie van zowel nieuw-gesynthetiseerd stanniocalcine als stanniocalcine opgeslagen in secretorische granula. In beide gevallen wordt de secretie van het opgeslagen stanniocalcine preferent gestimuleerd. Dit impliceert de aanwezigheid van minstens twee verschillende stanniocalcine-pools waarvan er een (het opgeslagen stanniocalcine) gestimuleerd wordt door een verhoging van de cytoplasmatische calcium concentratie, terwijl de ander (het nieuw-gesynthetiseerde stanniocalcine) minder afhankelijk is van deze stimulus. De door carbachol gestimuleerde stanniocalcine-secretie wordt daarentegen niet gekarakteriseerd door een preferente stimulatie van de secretie van opgeslagen stanniocalcine waardoor het onwaarschijnlijk lijkt dat stimulatie via een acetylcholine receptor gepaard gaat met een verhoging van de cytoplasmatische calcium concentratie.

stanniocalcine-secretie en kinetiek in zoet water en zeewater

De elektronenmicroscoop laat een duidelijke activatie zien van de lichaampjes van Stannius van aan zeewater geadapteerde vissen ten opzichte van die van aan het zoete water geadapteerde vissen. Deze activatie gaat echter niet vergezeld van veranderde stanniocalcine-concentraties in het plasma, hetgeen inhoudt dat de stanniocalcine-concentratie van het plasma niet altijd de secretorische activiteit van de lichaampjes van Stannius weerspiegelt en dat men ook rekening moet houden met het stanniocalcine-metabolisme. Uit onze kinetiek studies blijkt inderdaad dat het

stanniocalcine-verbruik, zoals bepaald door het meten van de "metabolic clearance rate", in zeewatervissen ongeveer twee maal zo hoog is als in zoetwatervissen (Hoofdstuk 6). Daarnaast zijn de "distribution-space" en de hypocalcemische potentie (het hypocalcemisch effect na injectie van een bepaalde hoeveelheid stanniocalcine) van stanniocalcine in zeewatervissen veel hoger.

Activatie van de lichaampjes van Stannius in zoetwatervissen die verplaatst worden naar zeewater is een langdurig proces dat meer dan 24 uur in beslag neemt. Het overbrengen van zeewatervissen naar zoet water reduceert de activiteit van de Stannius lichaampjes echter binnen 24 uur en resulteert in verlaagde calcium- en stanniocalcine-concentraties in het plasma. Deze verlaging van de stanniocalcine-concentratie is waarschijnlijk te wijten aan een abrupte reductie van de stanniocalcine-secretie gecombineerd met een (nog) niet verlaagd stanniocalcine-metabolisme (Hoofdstuk 4, 6).

De calciumconcentratie van het water kan variëren van ver beneden die van het vissebloed in zoet water tot ver boven die van het vissebloed in zeewater. Een zoetwatervis zal daarom meer behoefte hebben aan een hypercalcemisch hormoon terwijl een zeewatervis meer behoefte heeft aan een hypocalcemisch hormoon. Uit eerder onderzoek is inmiddels gebleken dat prolactine, het meest waarschijnlijke hypercalcemische vissehormoon, inderdaad een meer prominente rol vervult in zoetwatervissen en daarom een typisch hormoon voor het zoete water is.

De hoge synthetische en secretorische activiteit van de lichaampjes van Stannius alsmede de verhoogde calcium-regulerende potentie en het verhoogde verbruik van stanniocalcine wijzen op een grotere rol van het stanniocalcine in de handhaving van de calcium homeostase in zeewatervissen dan in zoetwatervissen. De conclusie die hieruit valt te trekken is dat stanniocalcine een typisch calcium-regulerend hormoon voor zeewater is.

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Je voudrais aussi remercier vivement Madame N. Mayer-Gostan de son amitié chaleureuse et de sa connaissance professionnelle excellente qui ont attribué à faire de mon séjour à Nice un grand succes et un vrai plaisir.

CURRICULUM VITAE

Robert Gerard Jules Marie Hanssen werd op 30 april 1960 geboren te Maastricht alwaar hij in 1979 aan het St. Maartenscollege het VWO diploma (Atheneum B) behaalde. In datzelfde jaar werd een aanvang gemaakt met de studie biologie aan de Katholieke Universiteit Nijmegen. Deze studie werd in 1986 afgesloten met het behalen van het "oude stijl" doctoraal examen met als hoofdvak Dierfysiologie (Prof. Dr. S.E. Wendelaar Bonga) en als bijvakken Chemische Cytologie (Dr. F. Wanka) en Cytologie/Histologie (Dr. W. Eling). Tevens werd in 1983 het diploma stralingshygiëne niveau 3 behaald. Vanaf december 1986 tot december 1990 was hij werkzaam als assistent in opleiding bij de afdeling Dierfysiologie van de Katholieke Universiteit Nijmegen. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht en werd een bijdrage geleverd aan het kandidaats- en doctoraal-onderwijs voor biologiestudenten. Naast deze werkzaamheden werd in 1987 het diploma proefdierkunde behaald. Bovendien werd in 1990 een drie maanden durend werkbezoek gebracht aan het Laboratoire de Physiologie Cellulaire et Comparée van de Université de Nice (Frankrijk).

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STELLINGEN

1. De concentratie van een hormoon in het bloedplasma van een vis weerspiegelt niet onder alle condities de activiteit van de klier die dit hormoon produceert.
Dit Proefschrift.

2. De hoge hypocalcemische potentie en plasmaklaring van stanniocalcine in zeewater geadapteerde palingen wijst erop dat dit hormoon veeleer een 'zeewater' dan een 'zoetwater' hormoon is.
Dit proefschrift.

3. Alvorens de effecten van extracellulair calcium op de stanniocalcine-secretie *in vitro* te vertalen naar de regulatie van de afgifte van dit hormoon *in vivo* dient men eerst de fysiologische 'range' van het extracellulaire calcium te bepalen.
Contra Wagner *et al.* (1989). *Mol. Cell. Endocrinol.* **62**, 31-39.

4. Het uitblijven van een snel-regulerend effect op de stanniocalcine-secretie na een fysiologisch relevante verhoging van de calcium-concentratie in het plasma van de paling betekent dat een directe relatie tussen de calcium-concentratie in het plasma en de afgifte van een calcium regulerend hormoon, naar analogie van de situatie in zoogdieren, in vissen onwaarschijnlijk is.
Dit proefschrift.

5. Ondanks eeuwenoude Brits/Franse tegenstellingen symboliseert de door beide volken volmondig ondersteunde uitspraak "Nice is nice" zowel op het sociale als op het taalkundige vlak de naderende Europese eenwording.

6. Leven als god in Frankrijk is onlosmakelijk verbonden met de "joie de vivre" die het Franse volk etaleert.

7. Niet alleen het bereiden maar ook het genieten van een goede maaltijd is een kunst die door de toenemende consumptie van diepvriesmaaltijden aan waarde dreigt in te boeten.

8. De hoedanigheid van Herwig van Hove als fysicus en gastronoom bewijst dat een academische en een culinaire carrière zeer wel samen kunnen gaan.